Protein Mediated Attachment Mechanisms Associated with Blastocyst Implantation

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DEDICATIONS

To the three people who have most influenced me, and unconditionally supported me in this endeavor. To my father, who taught me the values of hard work, a positive attitude, attention to detail, and that no task is impossible if you know how to improvise. To my mother, who taught me perseverance, a love of learning, and the real meaning of commitment.

I also dedicate this thesis to my wonderful husband and soul mate, Marc, who encouraged me to follow my dream and supported my pursuit with all the love, and emotional, psychological and financial support that I needed. I could not have done it without you!

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ABSTRACT

Protein Mediated Attachment Mechanisms Associated with Blastocyst Implantation

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It is estimated that one in three pregnancies ends in miscarriage. Little is understood about the underlying protein/receptor interactions responsible for attachment of the blastocyst to the uterine epithelial tissue. Even today, *in vitro* fertilization (IVF) sustains only a 30% average success rate. A primary cause of implantation failure in IVF is thought to be problems with the *initial* attachment process. Recent evidence suggests that the interaction between L-selectin and its sulfated ligands is the initial mechanism responsible for the *initial* capture of the rolling blastocyst. Further, secondary strengthening attachment processes are thought to be mediated through integrin/ligand interactions. No suitable animal model exists for the study of these initial events and research on human tissue is not possible due to ethical constraints. In this work, a trophoblast cell model was established and validated for examining initial L-selectin mediated attachment events in a hormonally-regulated environment. Further studies included the determining the effects of hydrodynamic shear flow on L-Selectin regulation in the cell model, and stimulation with dexamethasone, a steroid frequently included in the protocol for oocyte retrieval in IVF. For the first time, the attachment strengths associated with L-selectin/L-selectin ligand interactions as well as integrin/ligand interactions have been quantified in a trophoblast/uterine epithelial cell model, providing information that may be relevant to the *critical* attachment

strengths necessary to achieve and maintain successful implantation. *Further insight into these complex interactions will enable a more comprehensive understanding of the necessary conditions for successful implantation, and may lead to clinically relevant treatments for promoting fertility in vivo as well.*

CHAPTER 1. BACKGROUND

It is estimated that one in three pregnancies ends in miscarriage [1]. While numerous interactions and genetic factors of both the fetus and the mother may be responsible for miscarriage, the critical initial event for pregnancy maintenance is successful attachment and continued adherence of the blastocyst to the uterine endometrium. Implantation failure is also clinically encountered during *in vitro* fertilization (IVF) treatments. IVF is an increasingly common and widely used infertility treatment. It is used to treat nearly all causes of infertility, including ovulatory, tubal, male-related and immunologic [2]. Though success rates for IVF treatment have increased steadily in recent years [3] the implantation rate remains one of the greatest impediments to successful reproductive outcomes in IVF [4]. In 2004 over 88,000 standard IVF treatment cycles, over 9,000 donor oocyte cycles and over 4,000 cyropreserved embryo cycles were completed. Despite fertilization rates of up to 60%, pregnancy rates per transfer of embryos to the uterus in 2004 reached only 42.5% [4].

IVF is a costly and time-consuming effort for physicians, staff and patients. The average self-pay costs for treatments are frequently in excess of $10,000 [3].

In addition to costs associated with repeat procedures when implantation fails to occur, the high probability of multiple births associated with successful implantation is an added insurance burden. The ultimate goal of IVF is the birth of a singleton liveborn infant, and the only way to achieve this goal is to move towards a single embryo transfer policy [5]. Though research continues in the quest to improve the

techniques of optimum embryo selection, and embryo transfer, [6]; [7-9], the results have not been dramatic. From 1996 through 2004, the singleton live birth rates for fresh–non-donor cycles increased 34%, from 17% in 1996 to 23% in 2004 [4]. Furthermore, even in 2004, 90 % of the IVF cycles still involved the transfer of 2 or more embryos, and up to 32% of these resulted in multiple births[4] In 2001, it was estimated that the cost per "normal" live birth was $56, 419 [10]. However, neonatal care for high risk infants has been reported to be as high as $800, 000 [11]. Infants born as multiples are almost always premature and have higher rates of low birth weight, cerebral palsy, developmental delays, birth defects and death [12]. Even twins, who usually survive, are hospitalized twice as long as singletons and have much higher medical costs over the first five years [12]. In a 2004 study, it was reported that the total duration of hospital admissions for twins and triplets were respectively twice and eight times that for singletons, once duration of life had been taken into account. Inpatient costs were significantly higher for multiple births than for singletons, with the cost differences concentrated in the first year of life [12]. Thus, the motivation to achieve high success rates with single embryo transfers in IVF is very great.

Many IVF implantation *failures* are believed to be due to problems with attachment of the blastocyst to the endometrium [13]. There are few current clinical interventions that directly address this etiology. Some promising treatments that have been reported include:

1. Norethindrone (synthetic oral progestin) treatment of women deficient in $\alpha_v\beta_3$ integrin increased implantation rates in IVF [14].
2. Improved pregnancy rates and improved pregnancy outcome following embryo culture, prior to transfer, in endometrial culture medium with a GM -CSF (Granulocyte Macrophage Colony Stimulating Factor) content greater than 130 pg/ml [15].
3. Improved quality of human embryos with the addition of HB-EGF (Heparin Binding Epidermal Growth Factor) in ART cycles [16].

   With the help of these and other techniques, patients, physicians and staff may realize greater success rates for their remarkable efforts. Ultimately, the ideal scenario would be a 100% implantation success rate per embryo, which would result in reduced out of pocket and insurance costs, reduced physical and emotional stress and a much reduced ethical problem with regard to extra embryos.

It is the goal of this work to augment the knowledge base pertaining to the process of human implantation. Specifically, these studies are directed at the molecular interactions that are thought to occur in the *initial stages* of implantation, namely *apposition and adhesion*.

1.1 The Implantation Process

In order to completely understand the process of implantation, it is important to be familiar with the structure and function of both the uterus and the fertilized egg, at

both the physiological level and the molecular level. The uterine wall consists of three layers: the perimetrium, the myometrium, and the endometrium [17](Figure 1).

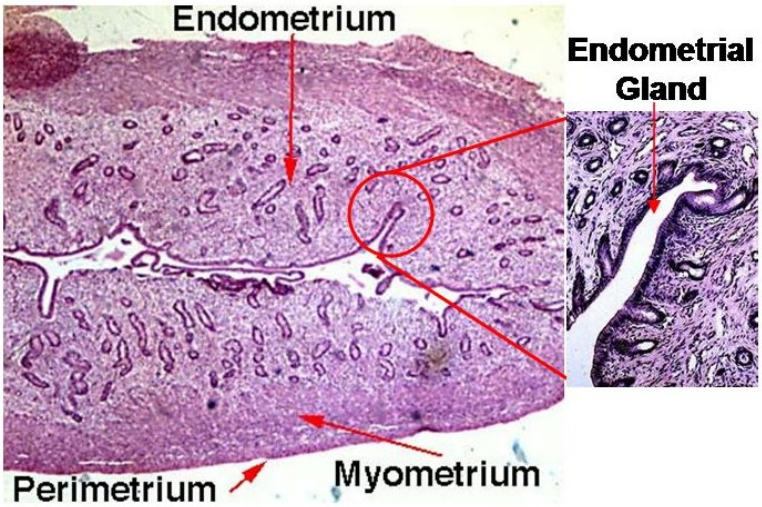


Figure 1: Three tissue layers of the uterine wall; with enlarged view of the endometrial gland [17].

The perimetrium, also known as the serosa, is the peritoneal covering on the outside of the uterus and it is composed of connective tissue [18]. The central layer is the myometrium, the thickest layer of the uterus. The myometrium consists of three poorly defined layers of smooth muscle. The third, inner layer, is the endometrium which consists of two layers differing in function and structure [18]. The functional layer, also known as the stratum fuctionale or functionalis layer is a thick apical layer which lines the lumen of the uterus. It is hormone responsive, and is covered with a simple columnar epithelium consisting of ciliated and non-ciliated cells [19]. The second layer of the endometrium is the basal layer, also known as the stratum basale,

or basalis layer. The functionalis layer represents the upper two thirds of the endometrium, and it is the site of tissue proliferation, secretion, and degradation, whereas the basalis layer comprises the lower one-third, and serves as a source of tissue regeneration [20]. It undergoes continuous morphological changes during the monthly cycle. The monthly menstrual cycle is on average, 28 days long. In figure 2 the phases of the menstrual cycle, the primary hormonal influences, and the corresponding progression of ovum as well as uterine development are shown.

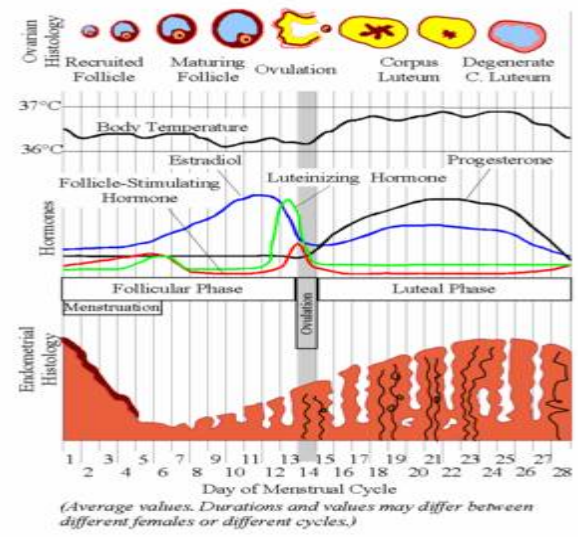


Figure 2: Phases of the Menstrual cycle showing hormonal influences, as well as ovum and uterine tissue development [21]

Menstrual cycles are counted from the first day of menstrual bleeding, because the onset of menstruation corresponds closely with the hormonal cycle. Following menstruation is the follicular or proliferative phase. During the follicular phase the

functionalis layer, stimulated by gradually increasing amounts of estrogen, regenerates as a result of new growth of glands, stroma and endothelial cells. Ciliogenesis, the appearance of ciliated cells around the gland openings, also occurs in response to higher estrogen levels [20]. Follicles in the ovary mature, and this phase ends with the release of an ovum or egg in an event called ovulation, which usually occurs around day 14. With ovulation, the corpus luteum forms and secretes progesterone, which acts on the endometrium to promote active secretion of glycoproteins and peptides into the endometrial cavity. The luteal or secretory phase begins after ovulation, and comprises days 15 to 28. Under the influence of progesterone, the endometrium undergoes changes to prepare for potential implantation of an embryo to establish a pregnancy. Pregnancy is most likely to occur during a small period of the secretory phase called the "window of implantation" or "window of receptivity" which occurs around days 16-19 in the monthly cycle. During conversion to the receptive state, the uterine epithelium undergoes not only a dramatic functional, but also a morphological transition [13]. Microvilli on the apical surface of the luminal endometrial epithelium fuse to form structures known as pinopods [22] which are histologic markers for the window of receptivity. Though their function has not been fully characterized, recent studies suggest that these progesterone dependent structures extract fluid from the uterus, and thereby facilitate closer contact between the blastocyst and the endometrium [20].

For successful implantation to occur, two temporally coordinated developmental processes must be achieved: the blastocyst must be capable of implantation, and the

uterus must develop to a receptive condition to support implantation [23]. The process begins with fertilization of the egg by the sperm within the Fallopian tube. As the growing embryo migrates towards the uterus, its cells keep dividing. The morula, a ball of embryonic cells, continues to divide while in the uterine cavity. During this time, a proteinaceous barrier known as the zona pellucida surrounds the developing embryo and its non-adhesive nature is thought to facilitate transport of the developing embryo to the uterus [13]. Within 72 to 96 hours of being fertilized, the embryo will enter the uterus. Around day 5, the embryo sheds its zona pellucida and at this point is considered "implantation competent" [24]. The embryo is now called a blastocyst, and it consists of two well-defined cell populations: the inner cell mass (ICM) and an outer cell layer called the trophectoderm. Approximately 6 days after fertilization, the blastocyst and maternal cells, two immunologically different organisms, enter into a paracrine dialogue or cross communication which initiates implantation. This process is described in three stages: apposition, adhesion and invasion [25]. During stage I, apposition, the implantation competent blastocyst becomes stationary, closely apposed to the uterine endometrium and the inner cell mass freely rotates in order to align itself in close proximity to the uterine endometrial epithelium [24]. In stage II, the adhesion phase, direct contact occurs between endometrial epithelium (EE) and the trophectoderm (TE). During the adhesion stage, "the association of the trophectoderm and the luminal epithelium is sufficiently intimate as to resist dislocation of the blastocyst by flushing the uterine lumen" [24]. In stage III, epithelial penetration and invasion occurs. This stage is defined by rapid proliferation

and differentiation of the trophoblast cells into an inner cytotrophoblast and an outer syncytiotrophoblast. Ultimately, cytotrophoblast cells become integrated into the maternal uterine wall creating a distinct two-way relationship between the blastocyst and maternal uterine luminal epithelium. Blood vessels of the embryo can interact with the maternal circulation which produces the formation of a functional placenta and pregnancy.

One of the fundamental requirements for the successful establishment and maintenance of pregnancy is the decidualization of the endometrium. Decidualization is defined as the differentiation of the fibroblast-like mesenchymal cells in the endometrium to a decidual cell which is morphologically and biochemically distinct [26]. Under the influence of progesterone, decidualization involves increased mitosis and differentiation of stromal cells. Also associated with decidualization is the progesterone-dependent infiltration of specific leukocyte subsets into the endometrial stroma, including natural killer cells, T cells, and macrophages [20]. The apical glycocalyx (the polysaccharide matrix excreted by epithelial cells forming a coating on the surface of epithelial tissue) decreases in both amount and negative charge character [25].

The effect of uterine contractility on the implantation process is a question which remains to be fully understood. Throughout the menstrual cycle, hormonally controlled wavelike activity patterns are known to occur in the uterus. These peristaltic contractions are initiated in the sub endometrial myometrium [27].

Estrogen is known to be the primary utero-stimulant. Estrogen levels peak in the pre-ovulatory period, with resulting contractions facilitating tubal ovum transport [28]. They appear to be *related* to successful pregnancies in both natural and assisted reproduction, but the exact role is not yet known. The relationship between contractile frequency and IVF outcomes is controversial, with some researchers reporting no correlation [29], and others reporting a negative correlation [30]. Furthermore, at the precise time of implantation, quiescence is thought to be beneficial. Bulletti states that "when implantation occurs, and the embryo interacts with the luminal epithelium surface, *the uterus must be relaxed, as this is a prerequisite for embryo nidation (implantation) and decidual invasion"* [31].

If pregnancy occurs, hormonal secretions from the placenta signal the corpus luteum to continue progesterone secretion, thereby maintaining the thick lining (endometrium) of the uterus, and providing an area rich in blood vessels in which the zygote(s) can develop. From this point on, the corpus luteum is called the *corpus luteum graviditatis*. However, if implantation does not occur, the corpus luteum will die, causing sharp drops in levels of both progesterone and estrogen. These reductions in hormone levels cause the uterus to shed the functionalis layer in menstruation.

1.2 Molecular Aspects of Implantation

After fertilization, the events leading to successful implantation of the blastocyst are highly regulated by crosstalk between the maternal and fetal tissues involving several endocrine, paracrine (signals which target only cells in the vicinity of the emitting

cell) and autocrine (signals which the target cell is the secretory cell itself) factors. The most important of these are the ovarian hormones, estrogen and progesterone. However, several other groups of molecules, such as cytokines, growth factors and cellular adhesion molecules have been found to be very important mediators of implantation mechanisms.

1.2.1 Hormones

Hormonal regulation of the implantation process has been extensively studied [20]. The dialog between embryo and receptive endometrium is under the control of the sex steroids, estrogen (estradiol, E_2) and progesterone, as well as other hormones, such as prolactin, calcitonin, and human chorionic gonadotropin (hCG).

The major factors that specify uterine receptivity are the ovarian steroids, progesterone and/or estrogens [24]. They initiate a cascade of paracrine and autocrine signal transduction which, via cell adhesion processes, will lead to the attachment and subsequent invasion of the embryo into the endometrium [32]. Estrogen and progesterone have two receptor subtypes: α and β, A and B, respectively. Estrogen receptor, (ER)- α is expressed by endometrial and stromal cells during the proliferative phase, but decreases during the secretory phase [33]. Endometrial expression of (ER)- β is limited to glandular epithelial cells [34]. The specific response of a cell to estrogen stimulation depends on the relative abundance of the ER subtype, the type of estrogen and the targeted response element [20]. Similarly, the relative proportions of progesterone receptor (PR)-A and –B within a target cell determine if gene activation will occur upon hormonal stimulation because

PR-A dominantly represses transcriptional activation by PR-B [35]. PR-A is expressed in the stroma and epithelium during the proliferative and secretory phases, however, epithelial levels of PR-A gradually decrease during the secretory phase [36]. PR-B is present in glandular and stromal nuclei only during the proliferative phase [37]. Progesterone receptor (PR) levels are increased by estrogens and growth factors, and decrease in response to progesterone [38]. ER- β also seems to down-regulate PR's in the luminal epithelium [39]. The down-regulation of PR during the window of implantation is *a prerequisite* for endometrial receptivity [40].

In addition to ovarian steroid hormones and local autocrine and paracrine signaling within the endometrium, *embryo-derived* signals seem to be essential for the establishment of a receptive endometrium and the initiation of the embryo-maternal cross talk. One of the first known hormonal signals of the embryo during implantation is human Chorionic Gonadotrophin (hCG). Its secretion begins no later than day 7 in the blastocyst stage which suggests a paracrine role of this hormone during the first steps of implantation in addition to its classical endocrine role in rescuing the maternal corpus luteum at the onset of pregnancy [41].

hCG is a glycoprotein that is synthesized by syncytiotrophoblasts, and principally serves to maintain corpus luteum progesterone function until the placenta is able to take over at 60 to 70 days' gestation [20]. The recent discovery of chorionic gonadotrophin/lutenizing hormone (CG)/LH receptor in the human uterus as well as LH receptor up-regulation during the period of endometrial receptivity has led to much interest in the potential direct role of hCG in implantation [42]. In stromal cells,

hCG promotes decidualization in the presence of estrogen and progesterone as determined by the increased transcription of prolactin, a marker of such differentiation. [43].

Prolactin (PRL) is a peptide hormone secreted in significant part by the anterior pituitary. The endometrium is one of the first extra pituitary sites that have been reported to synthesize and secrete PRL [44]. It is synthesized in the secretory phase of the menstrual cycle. Endometrial PRL (e-PRL) is similar to hypophyseal (from the pituitary gland) PRL in chemical, biological and immunological properties [45]. It has been hypothesized that progesterone is essential for the initiation of e-PRL synthesis and the maintenance of its production [46]. The effects of PRL are mediated by a membrane bound receptor, PRL-R. The coordinated temporal pattern of expression of e-PRL and PRL-R in non-pregnant and pregnant endometrium suggests that e-PRL may have an important role in the process of implantation and maintenance of pregnancy [45]. Some authors have suggested a role for e-PRL in the process of implantation by modification of the immune environment of the endometrium or by regulation of the factors within the glandular secretions that may control trophoblast proliferation and endometrium invasion [47, 48]. In a recent pilot study by Garzia et al, it was found that women with unexplained infertility and recurrent miscarriages present a defect in endometrial PRL production. It was suggested that e-PRL may be more than a simple marker of decidualization, but rather an active cytokine and that the endometrial defect may be associated with an

impairment of CD56+ NK killer cell endometrial recruitment ultimately resulting in unsuccessful embryo implantation and inadequate placentation [45].

Calcitonin is a 32 amino acid peptide hormone. Its most well characterized physiological role is to regulate calcium in bone and kidney cells [13]. However, recent studies have revealed that calcitonin is transiently expressed in the rat uterine epithelium overlapping the window of implantation [49]. Calcitonin was shown to be expressed in the *human* endometrium during the implantation period [50]. Calcitonin mRNA was found to be expressed in the uterine mucosa during the mid-secretory phase of the menstrual cycle, with maximal expression occurring between days 19–21, which coincides with the implantation window. The same authors reported that calcitonin gene expression in the human endometrium is induced by progesterone. Immunoreactivity for calcitonin mRNA was not detected during the proliferative and ovulatory phases in human endometrium, suggesting that the synthesis of this hormone is increased only during the implantation period [50].

1.2.2 Cytokines

Cytokines are small multifunctional glycoproteins that mediate communication between cells [51]. Cytokine is a general name: other names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action). In the human endometrium they play a major role in

reparative and inflammatory-like processes which occur every menstrual cycle, but they are also implicated in critical reproductive events such as ovulation and implantation [52]. The cytokines which have been identified as important to the implantation process include leukemia inhibitory factor (LIF), interleukin-1, (IL-1), IL-6, IL-11, Tumor Necrosis Factor-α (TNF- α), Colony Stimulating Factor- 1 (CSF-1) [51] and epidermal growth factors (EGF) such as Transforming Growth Factor-β (TGF-β) [53], and Heparin-Binding Epidermal Growth Factor (HB-EGF). Recently, genetic expression of IL-8 has been reported to be upregulated in an in-vitro co-culture cell model of human endometrial stromal cells and first trimester trophoblast cells, indicating a possibility of this protein's involvement in the implantation process as well [54].

The IL-6 family of interleukins, consists of IL-6 itself , IL-11, and LIF (Leukemia Inhibitory Factor), all of which are considered to have a role in the implantation process. LIF and IL-11 bind to ligand specific receptors, LIFR and IL-11R, respectively, and share gp130 (glycoprotein 130) as a signal transduction partner [55]. However, studies using IL-11Rα mutant mice have shown that IL-11 is crucial to decidualization, but not for the attachment reaction [56]. LIF was originally identified by its ability to induce the macrophage differentiation of the myeloid leukemic cell line, M1 [57, 58]. LIF is expressed with a molecular weight ranging from 38 to 67 kDa [52]. LIF has been linked to the implantation process since Bhatt and researchers reported a "dramatic and transient burst of LIF expression in the endometrial glands of the mouse uterus which is then reduced once implantation is completed" [59]. Studies with mice lacking a functional LIF gene showed that

embryos failed to implant, indicating that maternal LIF is *essential* for successful pregnancy. However, implantation failure was partially blocked by administration of LIF in the peritoneal cavity of the LIF deficient mice [60]. Thus, in the mouse, it has been suggested that endometrial LIF has either a paracrine effect on the embryo, or an autocrine effect on the endometrial epithelium to allow implantation [60].

LIF is also expressed in the human endometrium with patterns suggestive of a role in implantation [51]. It is regulated by other cytokines and steroid hormones, particularly progesterone. It has been hypothesized that the embryo, through hCG secretion, may actively participate in the control of endometrial LIF expression. LIF treatment of embryos has demonstrated a beneficial effect in the number of embryos that progress to the blastocyst stage [61]. Sawai, et al showed that that LIF increased hCG production and that the effect of LIF on trophoblast differentiation could be blocked by anti-hCG antibodies [62]. The key role of LIF in human implantation has been established based on abnormal LIF levels in infertile patients. Lessey, et al. reported that LIF is present during the window of implantation in women, and expression may be decreased in the endometrium of some women with infertility [63]. Endometrial LIF concentration was found to be lower in infertile women with recurrent embryo transfer failure after IVF [64]. Furthermore, Geiss, et al have shown, by screening for LIF gene mutations in fertile women and those with unexplained infertility, that heterozygosity for a LIF gene mutation could give rise to decreased availability or biological activity of LIF in the uterus and cause implantation failure [65]. Since LIF and its receptors, LIFR and gp130 exist in both

soluble and membrane bound forms, and soluble forms of these two receptors antagonize the actions of their ligands, a complexity of the LIF signaling pathway is implied [66, 67]. Dey has suggested dual roles for LIF, first in the preparation of the uterus, then in the attachment reaction [24]. However, to date, the molecular mechanism by which LIF executes its effects on implantation is not fully characterized.

IL-6 was originally identified as a factor inducing immunoglobin production in activated B cells. Within the human endometrium, IL-6 expression follows a regulated temporal pattern with highest levels detected during the luteal phase [68]. Strong immunoreactivity for IL-6 becomes detectable during the window of implantation [52]. The IL-6 receptor was found to be expressed by the blastocyst, the trophoblast and the endometrium [69].

Studies showing the regulation of IL-6 by steroid hormones have given conflicting results. Data showing the up and down regulation of endometrial IL-6 secretion by estrogen (E_2) and progesterone have been published [70, 71], [72] However, no direct effect of E_2 and/or progesterone treatment on endometrial IL-6 secretion by cultured endometrial epithelial cells (EEC's) could be established [72]. Another study showed that E_2 mediates the upregulation of IL-6 in immortalized EEC's, whereas E_2 and progesterone mediate up-regulation of its receptor [70]. It has therefore been speculated that, even if IL-6 is not directly regulated by E_2 and progesterone, the action of these hormones could be indirect via other mediators that are expressed at maximum concentrations in the late secretory phase [68]. Vandermolen, et al have

reported that IL-1β, a component of the IL-1 system (see below), stimulates endometrial IL-6 protein production in a time- and dose-dependent manner. Human endometrial IL-6 may therefore mediate some actions of IL-1β involving the endometrium and trophoblast [73].

IL-1 is a pro-inflammatory cytokine and one of the best characterized members of the cytokine family. IL-1 is produced in and secreted by the endometrium, and has a role in implantation.[14]. All components of the IL-1 system (IL-1α, IL-1, cell surface receptors IL-1R1, IL-1R2, and a receptor antagonist, IL-1Ra) have been examined at the maternal-trophoblast interface during implantation [74]. It is distributed in the human endometrium at both the protein and mRNA levels, and it is produced by decidualized and pseudo-decidualized stromal cells. [53]. In- vitro, IL-1 has been found to exert an influence on expression of the β_3 integrin (see section 1.2.4.1) subunit [75]. Embryo secretion of IL-1β is stimulated by endometrial factors [76, 77] demonstrating a clear interaction between the maternal endometrium and embryo prior to implantation. *In vitro*, studies have found that the release of IL-1β by cultured cytotrophoblast cells is directly proportional to their invasive capacity [78], and that IL-1β increases the production of matrix metallo- proteinase-2 (MMP-2) and -9 by the Jeg-3 trophoblast cell line [79]. It has been found by Arici et al to be a potent modulator of LIF expression in endometrial stromal cells in culture [80]. *In vivo*, it is therefore likely that decidual IL-1β may act in a paracrine manner by binding to IL-1R1 expressed by trophoblasts and regulating MMP-mediated invasion and hCG secretion [74].

Colony Stimulating Factor-1, (CSF-1), is a homodimeric protein that modulates proliferation, differentiation, and survival of numerous cell types, including fibroblasts, monocytes, macrophages and endothelial cells. It is one of the first examples of an implantation specific gene [81]. CSF-1 has been shown to be expressed by endometrial epithelial and stromal cells and by trophoblast cells, while its receptor, *c-fms*, is expressed on the embryo and placenta [82], [83]. Early studies in mice revealed that uterine concentrations of CSF-1 increase approximately 1000-fold during pregnancy, and there is a concomitant increase in messenger RNA (mRNA) in the luminal and glandular epithelium [84]. In humans, CSF-1 protein is much higher in the pregnant than the non-pregnant endometrium, and high in the placenta during pregnancy [85]. CSF-1 mRNA cannot be detected by Northern blot analysis in non-pregnant endometrium and this suggests that its role is not related to the early stages of implantation but rather to the processes involved in placentation. Furthermore, mice devoid of CSF-1 show impaired preimplantation embryo development, but can become pregnant and deliver viable litters [86].

A related protein, granulocyte macrophage colony stimulating factor (GM-CSF) has well characterized effects on survival, proliferation and differentiation of myeloid leukocytes and their precursors [87]. Similarly to CSF-1, GM-CSF is synthesized in the uterus by endometrial luminal and glandular epithelial cells in both mice and humans [88]. In the human endometrium, maximal expression of mRNA for GM-CSF is seen during the mid secretory phase, *coinciding with the window of implantation* [88]. The exact role of GM-CSF in the has not yet been elucidated,

however, a potential role in human implantation is suggested by reports of improved pregnancy outcome following embryo culture prior to transfer in endometrial culture medium with a GM-CSF content greater than 130 pg/ml [15]. Roles of the IL-1 family, IL-6, LIF and CSF-1 in implantation process are shown in Figure 3 [89].

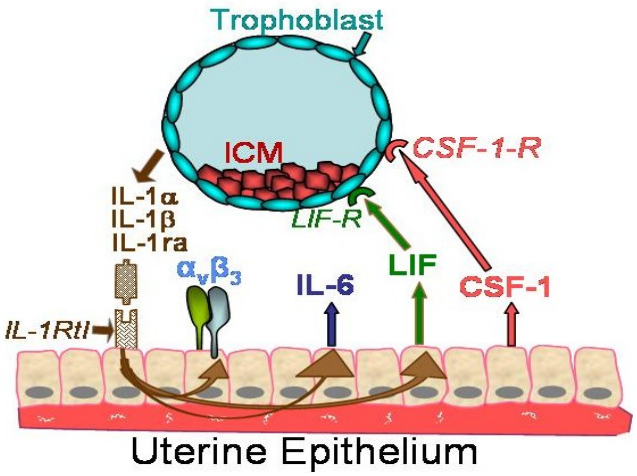


Figure 3: Important factors in the interaction of the blastocyst with the endometrium in the pre-implantation process: Embryonic secretion of IL-1 induces localized changes in the uterine epithelium, and the binding of IL-1 to IL-1Rt 1 (Interleukin 1 Receptor type 1) in the uterine epithelium may up-regulate the expression of αvβ3 integrins, IL-6 and LIF. Adhesion may be facilitated by the interaction between LIF, CSF-1 and corresponding embryonic receptors (Adapted from [89])

TNF- α (Tumor Necrosis Factor-α, Lymphotoxin B, Cachectin) was first identified in its 17 kDa secreted form, but further research showed that a non-cleaved 27 kDa precursor also existed in transmembrane form [90]. It was first isolated by Carswell et al. in 1975 in an attempt to identify tumor necrosis factors responsible for necrosis of the sarcoma "Meth A" [91]. In 1993 it was reported that human embryos secreted TNF-α in the medium until the morula stage, but not at the blastocyst stage [92].

Pampfer observed that the developmentally regulated expression of TNF-α receptors and its ligand in mouse embryos led to the down regulation of ICM (Inner Cell Mass) proliferation [93]. This selective inhibitory effect of TNF-α supports the hypothesis that this cytokine could contribute to the causes of many unexplained reproductive failures where high levels of TNF-α are present in the reproductive tract [94, 95]. In human first trimester villous trophoblast, TNF-alpha binding was found to be predominantly detectable on the syncytiotrophoblast and to a lesser extent on the cytotrophoblastic cells. Binding was not observed on adjacent embryonic or maternal cells. These results further support the idea that TNF- α may modulate early embryonic development and (later stages of the) implantation process [96]. TNF-α is inhibited by progesterone, which supports the hypothesis that it is *not* beneficial for pre-implantation embryo growth [97].

IL-8 (also known as Neutrophil Chemotactic Factor) is a pro-inflammatory cytokine which serves to attract neutrophils to the site of inflammation. During implantation a specific subset of leukocytes infiltrate into the endometrium from blood vessels and surrounding tissues [98]. Leukocytes also accumulate in the endometrial stroma during progesterone mediated decidualization [99].

Because the leukocytes do not possess steroid receptors, chemo-attractant cytokines, such as IL-8 seem to mediate their steroid dependant recruitment [100]. In the apposition phase of implantation, the blastocyst upregulates IL-8 at the protein and mRNA levels in endometrial epithelial cells (EEC's) [101]. Further evidence of this was seen in a recently published study by Popovici using comprehensive gene

profiling to analyze the effect of trophoblasts on endometrial stromal cells, during implantation [54]. In this work, an *in-vitro* co-culture system of endometrial stromal cells and first trimester stromal cells was established. After 24 hours of co-culture contact, cells were separated, and analyzed for changes in gene expression. The up-regulated genes included those for inflammatory response, immune response, regulators of cell growth and signal transduction and chemotaxis. IL-8 was found to be the most *significantly* upregulated gene, with a 367 fold increase. However, the pro-inflammatory effects of IL-8 seemed to be regulated [54].

When pregnancy occurs, the corpus luteum (CL) produces more progesterone, which maintains an inhibitory effect on IL-8, and inhibits leukocyte infiltration into the CL. This may represent an anti inflammatory effect of progesterone, suppressing the local inflammatory response at the time of implantation [102]. Thus, even though the process of implantation entails inflammation like events, exaggerated inflammatory responses may perturb the integrity of endometrial function and lead to pathological conditions, including abortion and complicated pregnancies, such as preeclampsia and underdevelopment of the fetus [103].

1.2.3 Growth Factors

The expression of various growth factors and their receptors in the uterus in a temporal and cell specific manner during the pre-implantation period suggests that these factors are important for implantation [24]. Heparin Binding Epidermal Growth Factor (HB-EGF), is one of several EGF-like molecules that are expressed in during or in close proximity to the window of implantation. HB-EGF has been found

to be expressed by the human endometrium, in secreted as well as membrane bound form, immediately before the window of implantation [104, 105]. Its expression is maximal during the late secretory phase (cycle days 20-24). In mice, during the receptive phase, HB-EGF is expressed at the apical surface of the luminal epithelium, and as such it has been proposed to be an attachment ligand in human implantation [104]. The membrane bound protein binds to the embryonic EGF receptor Erb-4, and may facilitate embryo attachment in mice [106]. In fact, cells expressing the membrane bound form of HB-EGF adhere to human blastocysts displaying cell surface ErbB4 [107]. In humans, addition of HB-EGF improves the quality of embryos in ART cycles [16] and may be a paracrine factor that stimulates other markers of uterine receptivity in human endometrium [81]. Thus, collective evidence suggests that HB-EGF has a significant role in preimplantation embryo development and implantation as a paracrine and/or juxtacrine factor [24].

The transforming growth factor β (TGF β) family comprises at least 42 distinct mammalian dimeric proteins that share a similar structure [108]. The TGF β family, along with its numerous receptors and signal transducing proteins is a highly complex system, and it has been found that disruption of any one member of the family by gene targeting in mice *has not* resulted in disruption of implantation [74]. TGFβ's may play a role in human implantation via their stimulation of fibronectin or vascular endothelial growth factor (VEGF) production [109, 110] or by promotion of adhesion of trophoblast cells to the ECM (extra cellular matrix) [111]. Furthermore, TGF β inhibits production and/or secretion of hCG, human placental lactogen,

progesterone and estradiol from trophoblast cell lines [112-114]. These *in-vitro* studies may provide some insight into the functional *in-vivo* actions of TGFβ, where the full repertoire of modulators are present, but this remains to be determined [74].

1.2.4 Cellular Adhesion Molecules (CAM's)

There are four major classes of cell adhesion molecules: integrins, cadherins, immunoglobins and selectins. The classifications are dependent upon the interactions which they undergo. Cadherins are dependent on calcium (Ca^{2+}) ions to function, hence their name. Immunoglobulins (antibodies) are proteins produced by plasma cells. They are designed to control the body's immune response by binding to substances in the body that are recognized as foreign antigens. Integrins are the primary receptors responsible for the attachment of cells to the extracellular matrix (ECM), though they also participate in cell-cell adhesive interactions. Selectins are a family of cell surface molecules found on both leukocytes and endothelial cells. All selectins are single-chain transmembrane glycoproteins, and they mediate cell to cell adhesion through protein-carbohydrate interactions. *Both selectins and integrins are considered to play major roles in the apposition and adhesion stages of implantation.*

1.2.4.1 Integrins

Integrins are involved in many physiologically important processes including embryological development, orienting cells in their environment, establishing their shape and polarity, haemostasis, thrombosis, wound healing, and immune and non-immune defense mechanisms [52]. They are heterodimers containing two distinct

chains, called the α (alpha) and β (beta) subunits. All integrins are made up of a combination of each of these subunits. In mammals, 20 α and 8 β subunits have been characterized. The molecular mass of the integrin subunits can vary from 90kDa to 160kDa. The α and β subunits are transmembrane polypeptides which associate with each other noncovalently to form two binding sites: one for the ligand on the outer membrane surface and a receptor site on the cytoplasmic side of the membrane. Integrins couple the ECM (extra cellular matrix) outside of a cell to the cytoskeletal filaments inside the cell Integrins themselves have no enzymatic activity, and therefore must rely on accessory proteins for generation of cytoplasmic signals [115]. They participate in two types of signaling. *Outside-in signaling* occurs when the binding of the ligand to the integrin activates classical intracellular signal transduction pathways to initiate cellular events. For example, in response to ligand binding, integrins aggregate into "focal adhesions sites", which leads to the recruitment of a network of cytoskeletal proteins such as α-actinin, talin and vinclulin [116] as well as intracellular signaling complexes; primarily kinases such as focal adhesion kinase (FAK), integrin linked kinase (ILK), and mitogen activated protein kinase pathway (MAPK) [117]. *Inside-out signaling* is the mechanism by which a cell regulates the affinity state of its integrin receptors [115]. Integrins can exist in inactive, partially activated and highly activated states. Inside-out signaling results in conformational changes in the external domain of the receptor. Mutational analysis suggests that the cytoplasmic domains of both α and β subunits may play a role in the modulation of integrin-ligand binding affinity [118].

Which ligand in the ECM that the integrin can bind to is dictated by the specific α and β subunits that it consists of. Examples of common integrin ligands are fibronectin, vitronectin, collagen, and laminin. The integrin binding domains interact with components of the ECM through recognition of an RGD (Argenine-Glycine-Aspartic acid, Arg-Gly-Asp) tripeptide sequence which was first found in the integrin ligand, fibronectin. This RGD sequence has also been identified in vitronectin, fibronectin, and osteopontin [118]. Figure 4 illustrates the interactions of integrin subunits with each other, as well as with the ECM [119].

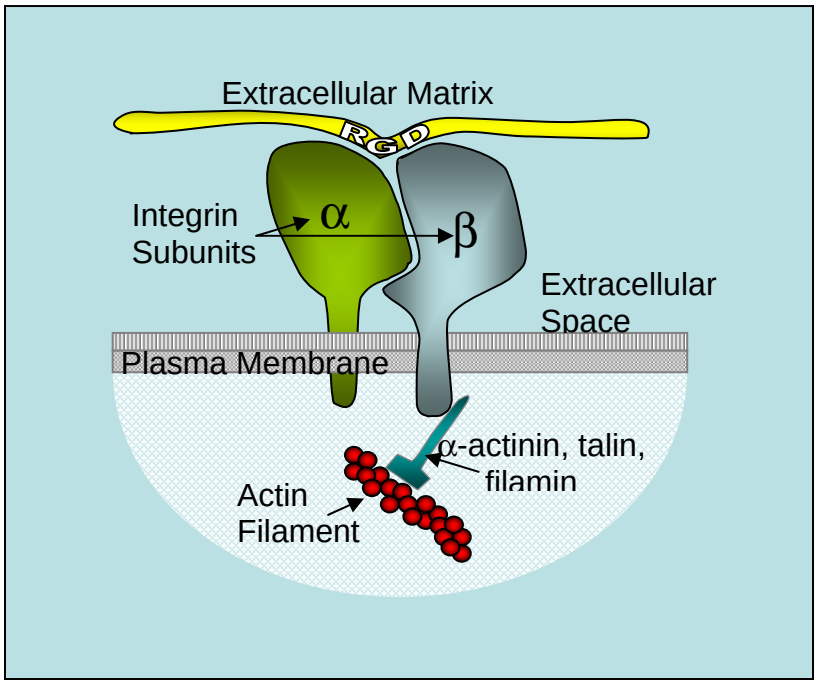


Figure 4: *Interactions of Integrin Subunits (Adapted from[119])*

Integrins are thought to play a significant role in implantation. They were first found in the endometrium by Lessey, et al. in 1992 [120]. The presence of integrin subunits on the human uterine endometrium throughout the menstrual cycle has been characterized through immunohistochemistry [120-122]. There are several integrins which are constitutively expressed by the uterine epithelium and these include: $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_5\beta_1$ [120]. Several integrins, however undergo cycle dependent expression during the menstrual cycle. In particular, the integrins which have been detected around the window of implantation are: $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_v\beta_3$ [81]. These integrins are receptors for collagen, fibronectin and vitronectin, respectively. Lessey has also reported that expression of these integrins occurs *only* during the window of implantation, on days 20-24 of the menstrual cycle [123].

Endometrial $\alpha_4\beta1$ binds to an alternatively spliced form of fibronectin, which is secreted by the trophoblast, and it is has been suggested to promote embryo attachment [124]. There is substantial evidence that the integrin $\alpha_v\beta_3$ is directly involved in the blastocyst/uterine epithelial adhesion process. This integrin binds to the RGD motif in fibronectin, osteopontin, vitronectin and laminin, among others [125, 126]. Oncofetal fibronectin is expressed by human trophoblasts [124], and $\alpha_v\beta_3$ is expressed apically in both mouse and human endometrial epithelium as is another α_v partner, β_5 which can bind to fibronectin and vitronectin [127]. In mice and rabbits, a functional blockade of $\alpha_v\beta_3$ integrin reduces implantation sites [128, 129].

In-vitro, immortalized ovine and porcine endometrial luminal epithelial (LE) and conceptus trophoblast cells abundantly express α_v and β_3 heterodimers at focal adhesion sites of cell anchorage to the basal substrate and these show evidence of integrin activation via osteopontin binding [130]. $\alpha_v \beta_3$, as well as osteopontin, have been detected by immunohistochemistry on the endometrial luminal epithelial surface, which is the location of blastocyst implantation [131]. The $\alpha_v \beta_3$ dimer is simultaneously present in the uterine epithelium and in the trophoblast. It is thought that the anchorage of the embryo is made possible through "loop interactions" [132]. Thus, $\alpha_v \beta 3$ expressed on the trophoblast cells recognizes endometrial osteopontin, and endometrial $\alpha_v \beta 3$ interacts with vitronectin and fibronectin expressed by the trophectoderm. In some women, the aberrant expression of $\alpha_v \beta_3$ is thought to be associated with certain infertility conditions, including endometriosis, luteal phase defect, hydrosalpinges and unexplained infertility [81]. In a study of 268 normal endometrial biopsies judged to be histologically "in phase", an absence of β_3 expression was found to be closely associated to the diagnosis of endometriosis [123]. Norethindrone (synthetic oral progestin) treatment of women deficient in $\alpha_v \beta_3$ integrin has been reported to result in increased implantation rates in IVF [14]. These examples support the hypothesis that $\alpha_v \beta_3$ is a critical integrin in the implantation process, and Lessey has proposed $\alpha_v \beta_3$ as a potential receptor for embryonic attachment [81].

Several studies have focused solely on the integrin *subunits* involved in implantation. Campbell, et al. demonstrated that six integrin subunits, α_3, α_v, β_1, β_3, β_4, and β_5 are

consistently expressed throughout the pre-implantation period [133]. Integrin subunits α_1 and α_4 are expressed in the endometrial epithelium during the luteal phase of the menstrual cycle. The subunit β_3 appears at day 20 of the cycle and co-exists with subunits α_1 and α_4 when the embryo is implanted [132]. Evidence has also been obtained for the presence of integrin subunits α_2, α_4, α_L, β_2 and β_7 on a small number of oocytes [115]. The α_v integrin subunit has been shown to be expressed in human embryos throughout early development from the two cell stage and up to the blastocyst [134]. In mice, at least three integrin subunits, α_2, α_{6A} and α_7 are newly expressed when the blastocyst becomes attachment competent [135]. The α_1 integrin subunit can only be detected after trophoblast outgrowth, which suggests that its expression is in response to contact with the ECM.

In mice, embryos carrying the homozygous null mutation for β_1 subunit will die shortly after implantation, due to degradation of the inner cell mass [136] however, the β_1-null trophoblast cells invade the uterine stroma, and survive longer than the inner cell mass, suggesting that integrins of the β_1 family are *not required* for initial implantation [115]. Furthermore, Yoshimura has reported that outgrowth, but not attachment, of embryos was inhibited in a dose dependent manner by an antibody that recognizes the β_1 subunit, suggesting that β_1 integrins are important for blastocyst development and differentiation *following attachment [137]*.

Thus, integrins have been known to be critical to the adhesion process in implantation since they were characterized by Lessey in the human endometrium in 1992. However, of all the integrins that have been characterized in the human reproductive

system, $\alpha_v \beta_3$ clearly stands out as the integrin most likely to be critical in the blastocyst attachment process.

1.2.4.2. Selectins

Selectins are type I membrane glycoproteins (carbohydrate binding molecules) which primarily mediate the docking of leukocytes to the blood vessel wall and the rolling of these cells along the endothelial cell surface [138]. Although selectins have been identified as being significant for the initial adhesion step in this process, their activity is known to be lost quickly due to rapid formation then breakage of adhesive bonds [139]. The selectins distinguish themselves from other adhesion molecules because they mediate *cell to cell bonding through protein-carbohydrate interaction*. In humans, three selectins have been identified and characterized: P-Selectin (named due to its initial discovery on blood platelets), E-Selectin (named due to its dominant presence on vascular endothelium) and L-selectin, (named because it was first discovered on blood leukocytes).

All three selectins contain many specific structural similarities : an NH_2 or amine terminal calcium (Ca^{++}) dependant C-type lectin domain followed by an epidermal growth factor-like (EGF) domain, a series of sequence consensus repeats (CR), a trans-membrane domain (TM) and finally a C- terminus cytoplasmic domain [138]. The three different selectins share an overall homology of 40–60% [140] with highest homologies in the lectin and EGF domains between 60% and 70% [140]. The major difference between L, E, and P-selectin is found to be the transmembrane and

cytoplasmic domains, and this suggests that they are probably regulated differently [138].

As with most proteins synthesized in the rough endoplasmic reticulum (ER), the selectins undergo “co-translational glycosylation” by the addition of a preformed precursor oligosaccharide composed of 14 sugars, consisting of N-acetylglucosamine, mannose and glucose. Glycosylation is the process of adding oligosaccharide side chains to the amino acids of a protein. The two major types of glycosylation that have been characterized within cells: N-linked and O-linked glycosylation. In N-linked glycosylation the oligosaccharide chain is attached to the amide nitrogen of an asparagine (Asn) residue which occurs in the tripeptide sequence Asn-X-Ser (Serine) or Asn-X-Thr (Threonine) , where X could be any amino acid except Pro (Proline). This sequence is known as a glycosylation *sequon*. *O*-linked glycosylation occurs at a later stage during protein processing, probably in the Golgi apparatus, during a process termed “Post-translational modification”, that is, the chemical modification of a protein *after* its translation. This occurs on the hydroxy oxygen of serine or threonine residues. Mucin type O-glycosylation (which is important in the production of L-selectin ligands) is initiated by enzymatic addition of N-Acetylgalactosamine(GalNAc) to serine or threonine by specific galactosaminyltransferase family of enzymes. Depending on which saccharide groups are subsequently attached to this first GalNAc residue, mucin O-glycans are divided into four major subtypes, or core structures. The core 1 structure is formed by addition of galactose to form Gal b1-3GalNAc-aSer/Thr. The core 2 structure

requires the core 1 structure as substrate and is formed by addition of N-Acetylglucosamine (GlcNAc) to form Galb1-3 (GlcNAcb1-6) GalNAc-aSer/Thr. The core 3 structure is formed by the addition of GlcNAc to form GlcNAc b1-3GalNAc-aSer/Thr. The core 4 structure requires the core 3 structure as substrate and is formed by addition of GlcNAc to form GlcNacb1-3 (GlcNAcb1-6) GalNAc-aSer/Thr.

Because the preformed oligosaccharide added to L-selectin during co-translational modification is transferred to the side chain amine (NH_2) group of an asparginine amino acid in the protein, it is said to be "N-linked". The diversity of the N-linked oligosaccharide structures that is seen on the mature glycoproteins is due to a later (post-translational) modification of the original precursor. Comparative structures of the three selectins are shown in Figure 5, along with proposed sites of glycosylation.

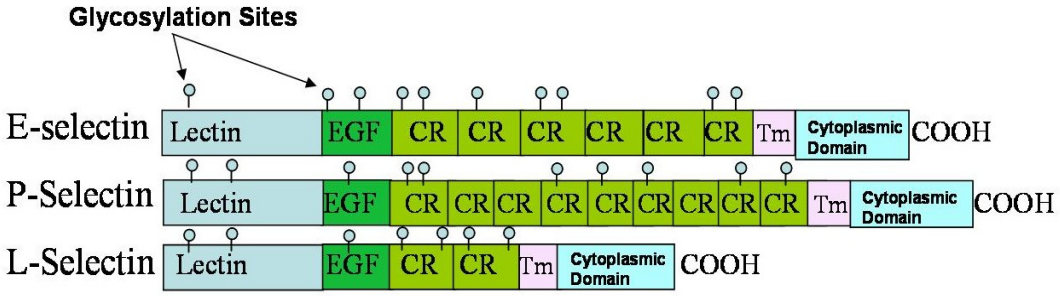


Figure 5: Comparative structures of human selectins where EGF = epidermal growth factor-like domain, CR =consensus repeats and TM = transmembrane domain

Though all three selectins play a major role in the cascade of molecular interactions that mediate leukocytes from the blood stream to the vessel wall, only one*, L-selectin* has been identified as a critical player in the capture and adhesion of the human blastocyst to the uterine endometrium in the complex process of implantation.

In 2003, a landmark study by Dr. Fisher's group showed that human trophoblasts expressed L-selectin, and that the initial attachment of the blastocyst to the uterine wall is mediated by oligosaccharide-based L-selectin ligands.. The ligand-receptor system was found to be functional, since trophoblasts could bind to ligand-expressing uterine luminal epithelium in tissue sections [141]. This important discovery supports what has recently been hypothesized as a clear parallel between the steps involved in the human blastocyst implantation process embryo-endometrial apposition, attachment and invasion, and leukocyte extravasion process: rolling/adhesion and invasion of the vascular endothelium [142]. The L-selectin adhesion system, as it applies to both of these processes is discussed in detail in the following sections.

1.2.5 The L-Selectin Adhesion System

This system is defined as the cellular adhesion protein, L-selectin, and its ligands, specifically those ligands which have been identified as important in the implantation process and ones which have a high level of O-linked glycosylation containing a sulfated sialyl Lewis *x* epitope.

1.2.5.1 L-Selectin Structure

L-selectin (CD62L, LAM-1, gp90^{MEL}, LECAM-1) was originally identified in mice in 1983, first by its affinity antibody, MEL-14, and then by its involvement in the

homing of lymphocytes to high endothelial venules of lymph nodes [143]. This protein, along with the other selectins were cloned and sequenced in 1989 [144]. L-selectin, the smallest molecule of the three selectins, is composed of 372 amino acid residues, corresponding to a molecular weight of ~37 kDa. However, the molecular weight of L-selectin is expressed in the range of ~70 and 110 kDa depending on the cell type. This could be explained by the fact that the DNA sequence of human L-selectin encodes a core protein of 37 kDa with eight possible sites for N-linked glycosylation. Thus, the observed different molecular weights are most likely generated by the glycosylation state [145]. The amino acid sequence for L-selectin has been elucidated, as well as the sequences that correspond to each domain structure (Figure 6).

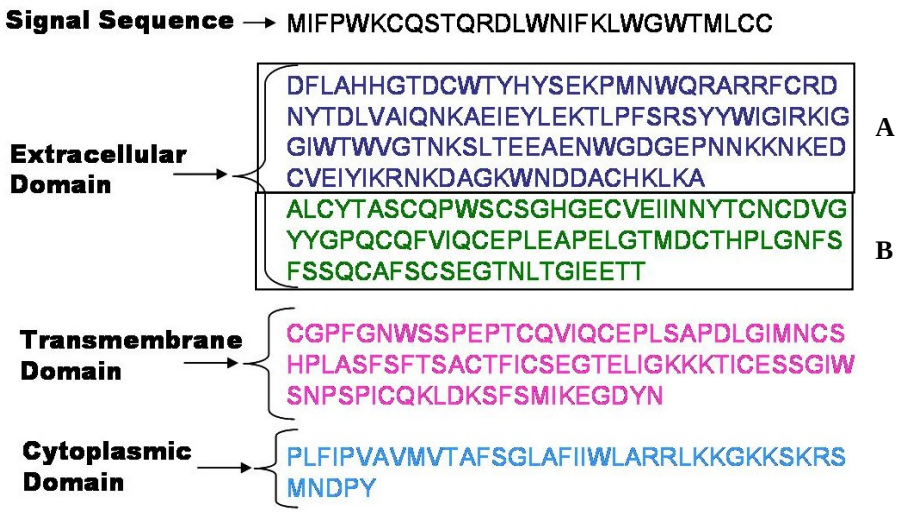


Figure 6: Amino acid Sequence of Human L-selectin (UniProtConsortium, 1991) with Domain Assignments: (A) *corresponds to the Lectin domain, and (B) corresponds to the EGF and CR domains.*

Figure 7 shows 3D filled and ribbon models of the lectin domain of L-selectin, as determined by homologous protein modeling (using the modeling program Blast [146], and in Figure 8, the, the spatial configuration of the entire L-selectin molecule is shown.

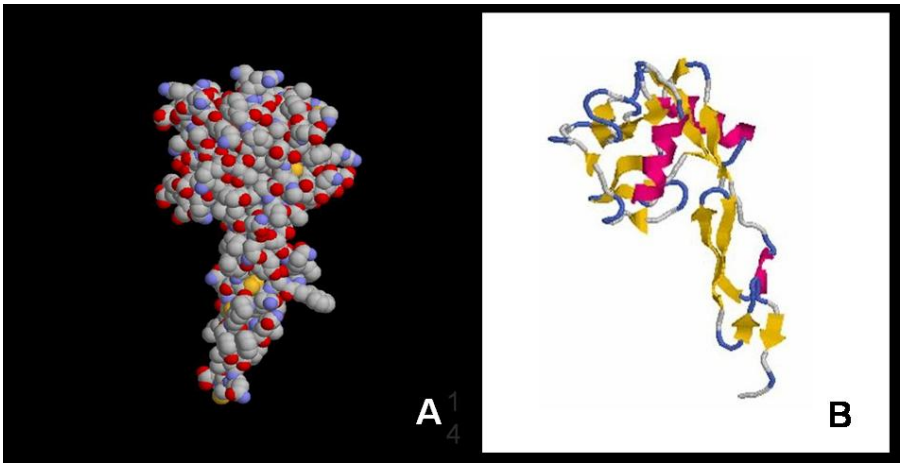


Figure 7: Proposed structure of lectin domain of L-selectin as depicted by RasMol:
A: Filled model showing negatively charged (red), positively charged (blue) and sulfur containing (yellow) amino acids
B: Ribbon model showing α helix (pink) and β sheet (yellow) sheet secondary structures.

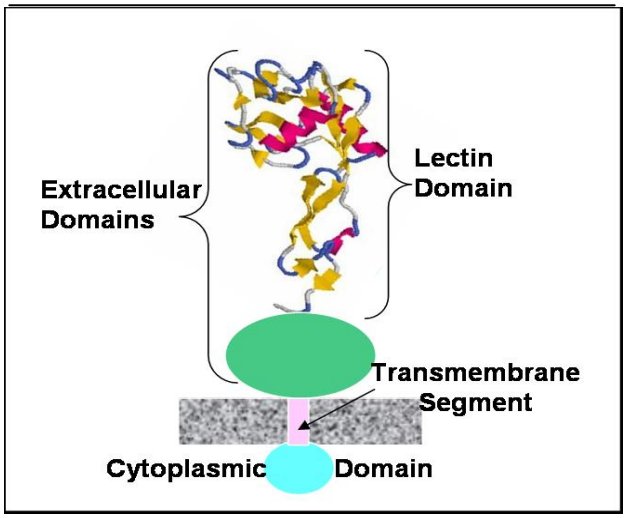


Figure 8: Spatial Configuration of the L-selectin Molecule

Kansas has shown that the *lectin* domain dictates its specificity of adhesion [147]. However, deletion of the EGF domain of L-selectin completely abolishes its adhesive function. This observation was interpreted to mean that the EGF domain is critical to the proper configuration of the lectin domain and hence its function, without being directly involved in ligand recognition. The adhesive function of L-selectin was found to be variably dependent on the CR domains [147].

It has also been shown that the cytoplasmic domain can regulate L-selectin shedding [148], and, through interactions with the protein calmodulin (CaM), can induce a conformational change in the extracellular domain that renders the cleavage site resistant to proteolysis [138].

1.2.5.2 Function and Regulation of L-Selectin

L-selectin function is regulated by a variety of mechanisms including gene transcription, post-translational modifications, and modifications of the L-selectin

topographic distribution by increasing or decreasing its availability at the cell surface [52]. Most of this information is derived from observations of vascular endothelial-leukocyte interactions. L-selectin is involved in the process of lymphocyte recirculation as well as in the migration of neutrophils and lymphocytes into inflamed tissues. Both up and down regulation of leukocyte L-selectin varies between different leukocyte sites and may be influenced by hormonal, cytokine, electrolyte or structural factors [144]. Given the recent hypothesized parallel between the steps involved in the human blastocyst implantation process of embryo-endometrial apposition, attachment and invasion, and the leukocyte extravasation process: rolling/adhesion and invasion of the vascular endothelium [142] it is instructive to review in detail the knowledge base regarding these interactions. Figure 9 shows the sequential steps involved in leukocyte extravasion through the vascular endothelium, with the multiple molecular interactions that have been shown to occur.

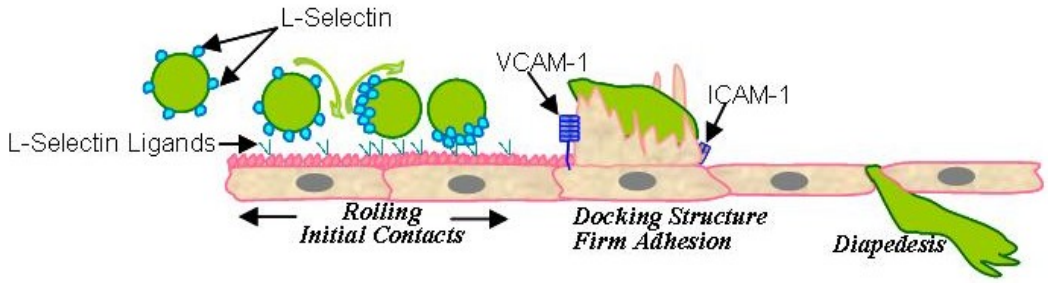


Figure 9: Sequential steps involved in leukocyte extravasation through vascular endothelium (adapted from Dominguez, 2005)

The recruitment of leukocytes from the flowing bloodstream is a rapid process. L-selectin is constitutively expressed on leukocytes, so the initiation or activation of the leukocyte-endothelial contacts is directed by the regulated appearance of its ligands (See section 1.2.5.5).

Following leukocyte activation, interactions between L-selectin and its ligands result in the capture or initial "tethering" of the neutrophil, and rolling along the blood vessel wall due to the coordinated formation and breakage of selectin-carbohydrate bonds [149]. L-selectin molecules are rapidly redistributed to form a cap at one end of the cell membrane during the rolling. During these events, L-selectin is shed due in part to metalloprotease activity which results in rapid accumulation of bioactive L-selectin in the blood [55]. Autoproteolysis of L-selectin can occur after exposure to various inflammatory mediators such as TNF-α, or lipopolysaccharide (an endotoxin) but it may also occur from normal rolling interactions with the vessel wall. Since metalloprotease inhibitors significantly reduce rolling velocity and cleavage of L-selectin, Walchek, et al. suggest that L-selectin is routinely shed from neutrophils under normal non-pathalogic conditions [150]. The dynamics of L-selectin rolling and shedding are discussed more fully in sections 1.2.5.3 and 1.2.5.4.

Cytoplasmic domains of bound and activated L-selectin and its ligand, PSGL-1 are linked to signal transduction pathways that lead to integrin activation [151]. Chemokines induce the high affinity conformation of leukocyte integrins that bind to the integrin receptors, ICAM-1 (CD54) and VCAM-1. Upon firm adhesion, mediated by the integrin-ligand interactions, the endothelial cells develop a 3-

dimensional docking structure that prevents the detachment of the adhered leukocyte, allowing it to proceed to diapedesis (extravasation).

Much is known about factors that down regulate L-selectin expression but less is known of factors that increase its expression and synthesis [144]. Steroids, such as dexamethasone or methylprednisone, modulate inflammation through effects on L-selectin expression. Methylprednisone decreases the expression of L-selectin in circulating neutrophils [152]. Dexamethasone decreases the *number* of circulating leukocytes expressing L-selectin in animals [153]. Several studies have demonstrated that *in vivo* administration of dexamethasone caused nearly complete downregulation of L-selectin on blood neutrophils [154, 155]. DeCoupade has shown that Annexin 1 (ANXA1), a calcium binding protein which is upregulated by glucocorticoids, mediates some of their anti-inflammatory properties through L-selectin shedding [156]. Data obtained in this study using the immortalized monocytic cell line, U 937, have shown that, following dexamethasone treatment, endogenous ANXA1 translated on the membrane, co-localized with L-selectin and possibly contributed to its shedding through protein/protein interaction [156]. Soluble L-selectin (sL-selectin), that which has been shed from the cell membrane, often retains functional activity, and may inhibit L-selectin mediated leukocyte binding to endothelium in-vivo [157]. During the course of an inflammatory response, the cytokine TNF- α triggers leukocytes to increase expression levels of L-selectin [158]. Non-Steroidal anti-inflammatory drugs (NSAID's) such as aspirin, indomethacin, diclofenac and ketoprofen inhibit neutrophil adhesion to TNF-α activated endothelium in-vitro under

non-static conditions, down regulate leukocyte L-selectin surface expression, and cause shedding [159]. As noted earlier (section 1.2.2), TNF-α is *not* considered to be a significant mediator in the implantation process, but is suspected to contribute to aetiology of many unexplained reproductive failures [160]. Thus, the role of TNF- α in the regulation L-selectin in the reproductive system may not be comparable to its role in the leukocyte adhesion process.

Interferon- α, a cytokine with multiple postulated mechanisms that is used as anti-tumor therapy in several diseases, upregulates L-selectin in lymphocytes, an effect which is partly dependent on inducing production of L-selectin mRNA [161].

Though early investigations focused on L-selectin expression under conditions of stasis, it has also been shown that hydrodynamic shear forces (fluid flow) alone can increase expression of L-selectin [162]. This very important effect could also be a mediator in the upregulation of L-selectin on trophoblast cells.

1.2.5.3 Effect of Fluid Flow on L-selectin Expression and Function

It has been demonstrated that the capture of leukocytes and subsequent rolling requires *critical thresholds* of shear stresses to occur [162-165]. This effect is not shared by the other selectins, but is unique to L-selectin. Furthermore, it appears to be an intrinsic property of the L-selectin molecule, as this dependence has been shown in cell-free immobilized L-selectin interacting with cell based or bead-immobilized L-selectin ligands [166, 167]. This effect is felt to be biologically important. Since L-selectin is constitutively expressed on blood neutrophils, shear threshold requirement may prevent inappropriate interactions with the blood vessel walls. [162]. The

threshold shear stress range required to support rolling and attachment has been quantified at 0.4 to 1.9 dynes/cm^2 [166, 168]. The rolling velocity can be controlled by two different mechanisms: intrinsic bond kinetics and the mechanical properties of the selectin-ligand bond [163]. Tethering is another feature that may occur as a result of interactions between selectins (or selectin ligands) with cytoskeletal proteins [139]. L-selectin mediated leukocyte rolling has been shown to be regulated by the cytoplasmic domain of L-selectin [167]. L-selectin is constitutively associated with the actin-binding protein, α-actinin [169] as well as with the regulatory cytoplasmic protein calmodulin [170]. Shortening or truncation of the 11 amino acid carboxyl terminal of the L-selectin cytoplasmic tail as well as disruption of the actin cytoskeleton led to the disruption of L-selectin association with α -actinin and abolished L-selectin rolling on inflamed venules under physiological shear flow, without altering carbohydrate recognition by the selectin [147]. These observations support the hypothesis that cytoskeletal anchorage of the L-selectin molecule reduces the sensitivity of its dissociation rate to increasing shear forces, a critical feature for mediating leukocyte rolling under physiological shear stresses [167].

In the presence of fluid flow, L-selectin has further been demonstrated to spatially redistribute, forming a "cap" or polarity of expression in the neutrophil membrane [171]. This group demonstrated that the capping of L-selectin, and PSGL-1, an L-selectin ligand, is facilitated by fluid shear, and dependant on MAPK (Mitogen Activated Protein Kinase) regulated membrane transport processes. They proposed that recruitment and assembly occur within membrane rafts [172] which are

microdomains within the cell membrane that have recently attracted attention, as they can serve as platforms for signaling processes. The capping of the L-selectin in neutrophils is thought to provide an important role in the mechanics of adhesion while the cells are in a flow environment. In particular, L-selectin binding and clustering at a single site of membrane contact involving few microvilli provides for local and rapid recruitment of activated β_2- integrin and efficient conversion from neutrophil rolling to arrest in shear flow [171]. This same effect is not seen under static conditions, where L-selectin remains homogeneously distributed throughout the neutrophil cell membrane.

The effect of fluid flow on *trophoblast* L-selectin expression has not been previously described. The embryo does not have a self propelling mechanism (e.g., cilia or flagella), thus it is passively transported by intra-uterine fluid. Fluid movements in the uterus may be induced by ciliary activity, myometrial contractions and mucin secretions by the endometrium [141]. Cilia-mediated flow has been shown to provide directional flow in the oviduct [173-175]. Accordingly, the characteristics of intra-uterine fluid flow pattern in the uterus play an important role in the process of embryo transport to the implantation site [176], but whether or not this affects the expression of L-selectin remains to be determined.

1.2.5.4 L-Selectin Shedding

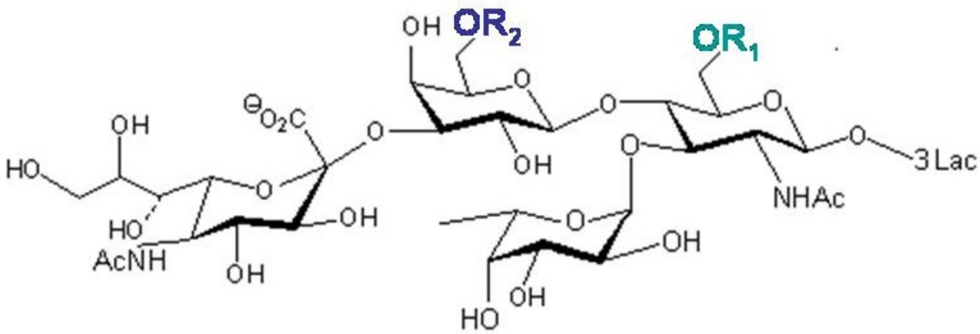
L-selectin shedding, i.e. cleavage of its ectodomain, plays an important role in the attachment of neutrophils to the endothelial cells of the capillary venules. While neutrophil capture is mediated by L-selectin sulfated carbohydrate side chains,

breaking the bonds is generally thought to be achieved by proteolytic cleavage of the L-selectin ectodomain from the leukocyte surface. The soluble cleavage product has retained activity and can inhibit lymphocyte attachment to endothelium [157]. Cleavage of the molecule occurs in a region that links the consensus repeats with the transmembrane domain (see figure 3) [177] thereby generating a 62-110 kDa soluble and a 6 kDa transmembrane cleavage product depending on cell type and glycosylation state [177-179].

There are several metalloproteases known to function as sheddases, among them metalloprotease 17 (ADAM17, TACE) [180-182]. Inhibition of L-selectin shedding in neutrophils can occur through synthetic hydroxamic acid-based inhibitors of MMPs, such as Ro 31-9790 and TIMP-3 [183]. L-selectin has been shown to shed in the presence of high flow (high shear stresses) in neutrophils [171]. L-selectin shedding is also an anti-inflammatory mechanism in neutrophils to downregulate the molecule from the leukocyte surface. In the U-937 monocytic leukemia cell line, which constitutively overexpresses L-selectin, shedding can be induced by the glucocorticoid, dexamethasone [156]. DeCoupade demonstrated that the L-selectin shedding is mediated by annexin 1, a protein which binds calcium and phospholipids and is abundantly found in granulocytes and monocytes [156]. Currently, little is understood about L-selectin shedding in trophoblasts. However, equilibrium between L-selectin expression and shedding may influence primary attachment strength of the blastocyst to the endometrium.

1.2.5.5. L-Selectin Ligands

Successful implantation is thought to depend on the *initial* interaction of the L-selectin expressing trophoblast, , and the uterine endometrium, which expresses oligosaccharide-based selectin ligands [184]. Thus, receptivity of the uterus may be critically influenced by the expression of L-selectin ligands. As with L-selectin, the discovery and characterization of the chemical and functional aspects of L-selectin ligands occurred within the realm of the vasculature, in particular, lymphocyte homing to secondary lymphoid organs. Early studies demonstrated that L-selectin recognizes a carbohydrate-based determinant, with an essential sialic acid moity [185]. These determinants are O-linked oligosaccharide side chains in a certain spatial conformation that seems to be an important feature for L-selectin ligand binding, and they typically contain terminal components that include α2,3-linked sialic acid and α1,3-linked fucose, typified by the sialyl Lewis x (sLe^x) determinant [139]. The dependence of L-selectin binding on *sulfation* of the ligand was first established by Imai in 1993 through the use of the metabolic inhibitors of sulfation: chlorate and brefeldin [186]. Subsequently, the recognition of the L-selectin ligands by MECA 79 antibody was shown to be sulfation dependent [187]. MECA-79 is an antibody which recognizes the *sulfated* sialyl Lewis X (sLe^x) epitope present in the ligands to which L-selectin binds. The structure of the sialyl Lewis *x* tetrasaccharide is shown in figure 10, with sites of sulfation [188].



A= 6-sulfo sialyl Lewis X: $R_1 = SO_3^-$, $R_2 = H$

B = 6'-sulfo sialyl Lewis X: $R_1 = H$, $R_2 = SO_3^-$

Figure 10: Sulfated forms of Sialyl Lewis x Tetrasaccharide[188]

MECA 79 has been shown to react with glycoproteins of several molecular weights. In human tonsil lysates, MECA 79 reactive proteins were seen at MW 65, 105, 160 and 200 kDa.[189]. In Peripheral lymph node HEV, seven major species were found to be reactive with MECA 79 in western blots, under both reducing and non-reducing conditions. These were located at molecular weights of 200, 170, 115, 90, 75, ~60 and 50 kDa [190]. The specific identification of these proteins remains to be fully elucidated, however, it has been proposed that the band at 160 may represent podocalyxin [185], the bands at 115, 105 and 90 may correspond to specific glycoforms of CD34 [185, 191, 192] the band at ~60 kDa may correspond to a subset of MadCAM-1 [187] and the band at 50 kDa may represent GlyCAM-1 [187, 192]. The band at ~60 kDa was not expressed strongly, and this is thought to be due to its being obscured by stronger binding of MECA 79 to GlyCAM -1 at 50 kDa [187].

Sulfation is a post translational modification, requiring the mediation of sulfotransferase enzymes. A major class of sulfotransferases which are involved in the post translational modification (sulfation) of L-selectin ligands is the "GlyNAc6ST" family which functions through 6-*O*- sulfation of N-Acetyl Glucosamine (GlyNAc) residues in glycoprotein bound oligosaccharides [193]. This class of sulfotransferases can be divided into two groups: those with broad distributions, and those with restricted distributions. Examples of the first group include: N-acetylglucosamine (GlyNAc)- 6-*O*-sulfotransferase-1 (GlyNAc6ST-1) [194] which has been found in the bone marrow, peripheral blood leukocytes, spleen brain, spinal cord, ovary and placenta [195] and GlyNAc6ST-4 which has been identified in the heart, spleen and ovary [193]. Examples of the latter group include: High Endothelial Cell-N-acetylglucosamine-6-Sulfotransferase (HEC-GlcNAc6ST) found in High Endothelial Venules [196], I- GlyNAc6ST which is preferentially expressed in the intestine [197], and C-GlyNAc6ST which was named due to its exclusive expression in the cornea [198]. The essential nature of sulfotransferase enzymes was demonstrated by Uchimura whose group showed that a major class of L-selectin ligands is eliminated in mice deficient in GlyNAc6ST-1 and GlyNAc6ST-2 [199].

L-selectin interacts with several counter-receptors, and five have been well characterized: Gly-CAM-1 (glycosylation-dependent cell adhesion molecule-1), a secreted molecule lacking transmembrane domain [140, 200], CD-34 (a type I transmembrane glycoprotein) [201], MadCAM-1 (mucosal addressin cell adhesion

molecule-1) which includes a mucin domain and Ig-like domains [190], PSGL-1 (P-selectin glycoprotein ligand-1) [202] and podocalyxin [191].

GlyCAM-1 (Sgp50) is a highly glycosylated serine and threonine rich protein, with a molecular weight of ~50 kDa. Structural analyses of GlyCAM-1 have revealed that ~70% of the MW of this glycoprotein is due to the carbohydrate component. GlyCAM-1 has been identified in lymphoid high endothelial venules, and in lactating mammary glands, where it is expressed in a hormone dependant manner [203]. Additionally, this ligand contains no transmembrane domain, and functions as a result of secretion directly into the blood [204]. It consists of a mucin–like scaffold, that presents a carbohydrate ligand(s) to the lectin domain of L-selectin [140, 205]. As a result of a study focused on the regulation of murine GlyCAM-1 in response to an inflammatory stimulus, it was suggested that released plasma GlyCAM-1 may trap, at least in part, L-selectin shed from stimulated leukocytes and neutralize it. Furthermore, these results suggested that upregulation of GlyCAM-1 may be initiated by the inflammatory cytokine IL-6 [204].

CD34, originally termed Sgp90, is a sialomucin-like glycoprotein which contains a transmembrane domain, and has been shown to be stably associated with the cell surface. [201]. The molecular weight of CD34, a heavily glycosylated protein, is ~116 kDa. As with GlyCAM-1, CD34 was originally detected in high endothelial venules (HEV) of peripheral lymph nodes (PLN), but its expression in human tissue is more extensive than GlyCAM-1. CD34 is expressed in non-lymphoid sites in the vasculature, where it is involved in L-selectin mediated leukocyte rolling and has

long been a surface marker for the purification of pluripotent stem cells for bone marrow transplantations [201]. For high endothelial venules (HEV) CD34 serves as a ligand for L-selectin, whereas CD34 is not the ligand for L-selectin in hematopoietic stem/progenitor cells (HSPCs) and in fact, ligands for hematopoietic CD34 remain to be identified [206] It has also been detected in capillaries of the human brain [207]. Two glycoforms of CD34 have been identified in both murine and human cells: a full length form and a truncated form. Their structures consist of identical extracellular regions, and differ only in their intracellular cytoplasmic domains [208]. The significance of this differentiation has not yet been established. The role of CD34 in the process of human implantation has not been explored to any significant extent. It is known that CD34 knockout mice have no detectable abnormality in leukocyte trafficking are viable and able to reproduce [208]. In 2002, Zhang, et al published the findings of a comprehensive study characterizing uterine endothelial cells throughout the menstrual cycle for several protein biomarkers thought to be involved in the normal function of the human endometrium, including CD34 [209]. There appeared to be an upregulation of CD34 in the secretory phase of the menstrual cycle. Though strong staining for CD34 was observed on small vessels and capillaries in the endometrium, as expected, an unexpected finding was the particularly strong staining seen in the stroma around the glands [209]. Since this work was performed before the discovery of the involvement of the L-selectin adhesion system in the process of implantation, the role of CD34 was only examined with regard to its association with menstrual disorders. However,

given the unexpected finding in this study, it would be interesting to explore the temporal regulation of CD34 expression on the uterine epithelium.

Podocalyxin (also called podocalyxin-like protein-1 or PCLP-1) is a protein that is expressed at high levels by podocytes (cells of the visceral epithelium in the kidneys), vascular endothelia, platelets and haemopoietic stem cells. It is a transmembrane mucin-like protein that possesses a structure very similar to CD34 and endoglycan. This protein, with a MW of 150-165 kDa consists of a mucin domain, a disulfide bonded globular domain, a transmembrane region and a highly charged cytoplasmic tail with potential phosphorylation sites for protein kinase C and casein kinase II [210]. It has been shown to be an adhesive ligand for L-selectin expressed by leukocytes, one of its major roles in the body [201]. Based on a number of studies, however, it has been hypothesized that podocalyxin can also act as an "anti-adhesion" molecule, due to its negatively charged mucin domain. [211-213]. Thus, in HEV's, podocalyxin, as well as CD34, is thought to serve two functions. Initially, these ligands provide tethers for L-selectin expressing leukocytes, and later, they have been observed to relocate, in response to cell activation, to cell junctions and act to "spread" the endothelial cells to facilitate leukocyte leukocyte transmigration. [210]. Little is known of the role of potential in the process of human implantation. Weak immunostaining for podocalyxin has been reported in the endometrial endothelium during the mid-secretory phase, and this has been associated with the selective recruitment of peripheral blood natural killer (NK) cells to the human endometrium as a normal function in the menstrual cycle. [214].

P-selectin glycoprotein ligand-1 (PSGL-1) is a dimeric mucin-like 120-kDa glycoprotein composed of 412 amino acids. This glycoprotein, despite its name, may serve as a ligand for all three selectins. PSGL-1 is a cell associated ligand, with an extracellular domain (consisting of two subunits), a transmembrane domain, and a cytoplasmic domain [215]. Each subunit of the extracellular domain contains 70 serine and threonine residues, which are potential sites for O-Glycosylation, as well as three potential sites for N-Glycosylation [215]. It has been shown that dimerization of the subunits (though not necessarily through covalent bonding) is essential for its high affinity binding to P-Selectin [215]. PSGL-1 displays appropriate sialyl Lewis *x* carbohydrate determinants required for L-selectin recognition [216]. The role of PSGL-1 as a ligand for P-Selectin has been widely studied [215, 217-219]. The role of PSGL-1 as an L-selectin ligand has been recognized more recently. As such, it has been well characterized in its role in increasing leukocyte recruitment to inflammatory sites by mediating neutrophil attachment to adherent neutrophils [220, 221]. During this process, activated neutrophils will shed their L-selectin, however, they will retain the PSGL-1 ligand [221].

The three selectin cellular adhesion proteins each have distinct requirements for PSGL-1 ligand sulfation. PSGL-1 contains tyrosine sulfate and sulfated carbohydrate binding sites. Binding of PSGL-1 to both L-selectin and P-Selectin is dependent on tyrosine sulfation of amino terminal residues 46, 48, and 51 and on α 1,3 fucosylation of core-2 *O*-Glycans attached to Thr-57 [222, 223]. The binding of E-Selectin however, does not require tyrosine sulfate residues [222, 224]. Kanamori,

et al demonstrated that under shear flow, selectin mediated leukocyte rolling was highly influenced the specific binding sites. Whereas L-selectin "prefers" carbohydrate 6-sulfation much more than tyrosine sulfation, P-Selectin favors tyrosine sulfation in the PSGL-1 molecule [225].

PSGL-1 expression has been shown to be regulated by granulocyte colony stimulating factor (G-CSF), dexamethasone, and Endotoxin [226, 227]. Endotoxin is a heat-stable polysaccharide like toxin bound to a bacterial cell membrane. It is responsible for many of the virulent effects of gram-negative bacteria. In healthy human volunteers, leukocyte activation by endotoxin challenge decreased the surface expression of PSGL-1 on monocytes and neutrophils and increased the level of soluble PSGL-1 (sPSGL-1) in plasma [227].

In vitro, neutrophils rapidly down-regulated PSGL-1 expression upon exposure to G-CSF as well as dexamethasone, and induced shedding of soluble PSGL-1. This finding supports the concept that the mechanism for down regulation of PSGL-1 is proteolytic cleavage [226]. As with the known effect of dexamethasone on L-selectin shedding [156], this effect may have implications with regard to the functioning of the L-selectin adhesion system in implantation, should PSGL-1 be identified as a specific L-selectin ligand in the uterine epithelium.

Mucosal vascular addressin cellular adhesion molecule-1 (MadCAM-1) is a cell surface mucosal endothelial adhesion molecule with immunoglobin and mucin-like domains. [190]. It was first described in 1988 as "a tissue-specific endothelial cell molecule involved in lymphocyte homing" [228]. It has been identified on high

endothelial venules (HEV) of Peyer's Patches, as well as the appendix, and mesenteric lymph nodes [228, 229]. MadCAM-1 is broadly expressed in the human embryo, starting from around week 7, and it gradually becomes localized to the mucosal vessels after birth [230]. MadCAM-1 has also been found to be expressed outside the endothelial cell lineage on fibroblasts and melanoma cells [231] as well as (weakly) in the brain and pancreas [229]. Animal model studies have shown MadCAM-1 to be present in the genital mucosa during chlamydial infection [232]. Generally, this molecule is expressed as a 55-66 kDa glycoprotein, however, a 40 kDa glycoform is also expressed by vascular endothelium [233]. The structure of MadCAM-1 consists of two extracellular amino terminal immunoglobin superfamily (IgSF) type domains that possess strong homology to the vascular adhesion receptors for leukocytes ICAM-1 (Intercellular Adhesion Molecule-1) and VCAM-1 (Vascular Cell Adhesion Molecule-1) [229]. Separating these domains from the cell surface is an extended mucin-like region of 115 amino acid residues followed by a transmembrane domain, and a 43 residue cytoplasmic domain [234]. MadCAM-1 is post-translationally modified with extensive O-linked glycosylation [235]. Structural studies with synthetically glycosylated MadCAM-1 have suggested that the glycosylation can contribute to conformational aspect of the protein, which can effect its stability and chemoenzymatic reactivity [236].

MadCAM-1 binds both the integrin α4β7, through its IgSF domains, as well as L-selectin, through its O-glycosylated mucin like region [234]. As such, it plays a role in both the capture *and* retention of leukocytes. The interaction of MadCAM -1

and α4β7 integrin has been shown to be the key step in lymphocyte homing to gut mucosa [237]. In the "adhesion cascade" in Peyer's Patches, the interactions of MadCAM-1 with α4β7 integrin occur after MadCAM-1 interactions with L-selectin, and prior to it's interactions another integrin, αLβ2 [238]. DeChâteau's group also studied the kinetic and mechanical properties of cell-substrate tether bonds in a shear flow environment with a focus on MadCAM-1 interactions with L-selectin and $\alpha_4\beta_7$. "Stable" tethering was defined as the binding of cells in flow to substrates that was followed by rolling or firm adhesion that lasted for >3 seconds (s). They discovered that stable tethering to MadCAM-1 was at a maximum under shear flow of 2 dynes/cm^2 or less, that the MadCAM-1-L-selectin bonds were slightly stronger and more stable than the MadCAM-1-integrin associations and that the rate of bond dissociation per second (k_{off}^o (s^{-1})) was greater for L-selectin interactions than α4β7 integrin interactions. This was explained by the fact that mechanically stable bonds are most important in early adhesive cascades, because a greater number of receptor-ligand bonds accumulate later in the adhesion cascade, and as such, the hydrodynamic shear force experienced by the cell is distributed over a larger number of bonds, therefore to be effective they may be less mechanically stable [238].

The regulation of MadCAM-1 expression has been studied, particularly with a focus on treatments for acute and chronic inflammatory conditions of the intestine, such as Crohn's Disease and ulcerative colitis [239, 240]. MadCAM-1 has been shown to be up-regulated in murine lymph node endothelium by the inflammatory cytokine, TNF-α, [241] and inhibited by dexamethasone treatment [242]. The up-regulation by TNF-

α is also blocked by IL-10 [242], and interferon-γ (IFN- γ) [241]. MadCAM-1 expression has not been characterized with respect to the uterine epithelium, where it would likely be involved in the process of implantation, but in a recent study of the temporal expression of L-selectin ligands in the human endometrium, MadCAM-1 was observed throughout the secretory phase, with the strongest staining seen in the late secretory phase [214]. This was associated with selective recruitment of natural killer (NK) cells to the endometrium as a normal function in the menstrual cycle.

1.2.6 Summary: Molecular Aspects of Implantation

The molecular aspects of human implantation are obviously very complex, and are likely to involve synchronized cross-communication between a receptive uterine endometrium and an implantation competent blastocyst. This process involves the hormonal regulation of numerous proteins and signaling pathways. Many "markers of uterine receptivity", that is, those which are present during the window of implantation, have been identified. The major factors that indicate uterine receptivity are the ovarian steroids, progesterone and/or estrogens. Progesterone, which is the dominant sex hormone in the secretory phase of the menstrual cycle, upregulates other hormones such as calcitonin and prolactin. Calcitonin mRNA was found to be expressed in the uterine mucosa during the mid-secretory phase of the menstrual cycle, with maximal expression occurring between days 19–21, which coincides with the implantation window. Calcitonin gene expression in human endometrium is induced by progesterone. The coordinated temporal pattern of expression of endometrial prolactin (e-PRL) and PRL-R in non-pregnant and pregnant

endometrium suggests that e-PRL may have an important role in the process of implantation and maintenance of pregnancy [45].

The expression of various growth factors and their receptors in the uterus in a temporal and cell specific manner during the pre-implantation period suggests that these factors are also important for implantation [24]. Two important growth factors that are considered markers of receptivity are: heparin binding epidermal growth factor (HB-EGF) and transforming growth factor- β (TGFβ).

Cytokines, normally play a major role in reparative and inflammatory-like processes which occur in the human endometrium every menstrual cycle, but they are also implicated in critical reproductive events such as ovulation and implantation [52]. The cytokines which have been identified as important to the implantation process, interleukin-1 (IL-1), and its receptors, IL-1R 1 and IL-1R 2, the IL-6 family of cytokines which includes IL-6, IL-11, and leukemia inhibitory factor (LIF), Colony Stimulating Factor- 1 (CSF-1) and the related protein, GM- CSF. IL-1R 1 expression in the uterus may up-regulate the expression of αvβ3 integrins, IL-6 and LIF in the uterine epithelium. CSF-1 has been shown to be expressed by endometrial epithelial and stromal cells and by trophoblast cells, while its receptor, *c-fms*, is expressed on the embryo and placenta. These processes are illustrated in figure 3.

One of the first known hormonal signals of the *embryo* during implantation is human Chorionic Gonadotrophin (hCG), which has a dual role in the reproductive process. Its secretion begins no later than day 7 in the blastocyst stage which suggests a paracrine role of this hormone during the first steps of implantation. Additionally, its

classical endocrine role is in rescuing the maternal corpus luteum at the onset of pregnancy [41].

The initial mechanical interaction between the blastocyst and the uterine endometrial tissue, termed apposition, is thought to be mediated by the cellular adhesion protein L-selectin and it's MECA-79 reactive ligands, which may include CD34, podocalyxin, GlyCAM-1 and MadCAM-1. Fisher et al. demonstrated the presence of L-selectin on the blastocyst, as well as a significant upregulation of the expression of the sulfated oligosaccharides that function as high-affinity counter-receptors for L-selectin on human uterine luminal and glandular epithelium during the window of receptivity [141]. L-selectin ligands are upregulated in the endometrium during the luteal phase coinciding with the high expression of L-selectin on trophoblastic cells. Finally, integrins, another classification of cellular adhesion molecules, are thought to play a significant role in implantation. Several integrins, undergo cycle dependent expression during the menstrual cycle, and are therefore considered to be important as markers of receptivity. In particular, the integrins which have been detected around the window of implantation are: $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_v\beta_3$.. These integrins are receptors for collagen, fibronectin and vitronectin, respectively.

With the discovery of the presence of the L-selectin on the human blastocyst and its ligands in the uterine epithelium at the time of implantation, parallel mechanisms, both mechanical and molecular, have been suggested for the process of leukocyte rolling, attachment and extravasation and the three stage process of implantation: apposition, attachment and invasion. The process of leukocyte extravasation is

illustrated in figure 9. Figure 11 illustrates the proposed molecular mechanism of implantation.

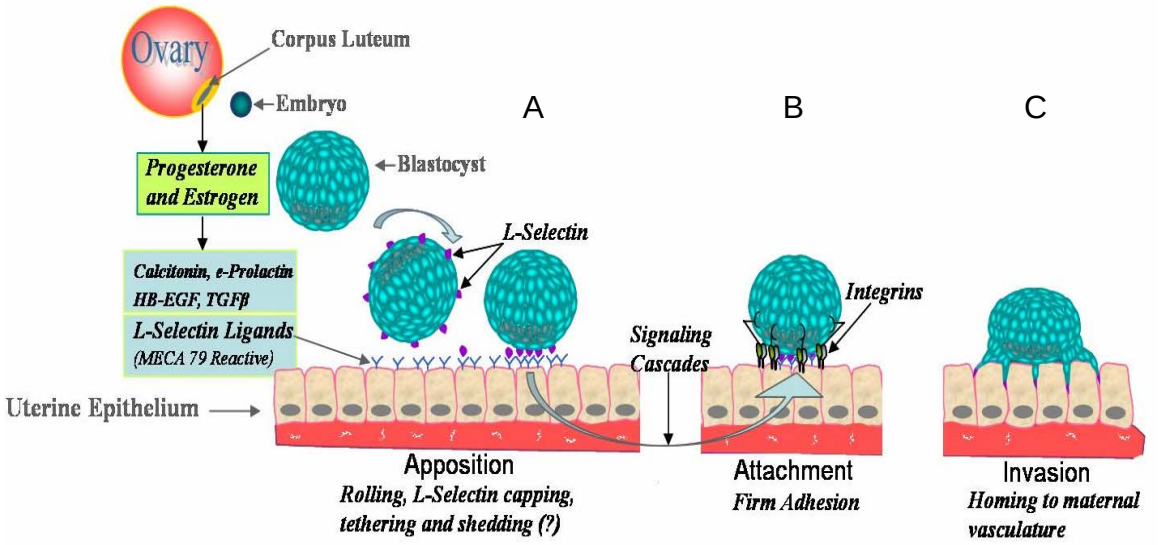


Figure 11: Proposed molecular mechanism of implantation

A) Progesterone and estrogen mediated up-regulation of other hormones, growth factors, L-selectin and its ligands, and primary attachment elicited through L-selectin mediated "tethering" of the blastocyst to the uterine epithelium, followed by signaling cascades of molecules (cytokines, MAPK's)

B) Integrin-ligand interactions facilitate stronger adhesion through secondary attachment mechanisms

C) Invasion of the trophoblast cells and formation of the fetal maternal vasculature.

1.3 Attachment Strength Measurements

Though the attachment strength values of the primary and secondary mechanisms have not been quantified in the blastocyst/uterine epithelial environment, the

neutrophil/vascular endothelial system has been. Approaches to attachment strength measurements vary considerably, depending on the cell-substrate system being characterized, the type of information desired, (quantitative or qualitative, etc), and the range of attachment strengths that are being measured. In vitro cell adhesion assays can be classified into static or dynamic models. Multiple examples exist in both categories, but those discussed in detail here are specifically applicable to the elucidation of adhesive interactions between L-selectin and its ligands, as well as integrin-mediated adhesion processes.

One of the simplest and most commonly used assays is the "static cell adhesion test" [243]. It consists of seeding a known number of cells onto appropriately prepared substrates, incubating in a static environment for a specified period of time, removing non-adherent cells by washing with physiological buffers, and quantifying the remaining adherent cells, through counting of a statistically significant sample [244-247]. The relative adhesion is determined as a percentage of the initial (seed) cell count. This approach provides qualitative observation of cell-substrate interactions, but it is neither quantitative nor reproducible because the cells are exposed to uneven, unknown detachment forces [248]. Additionally, the requirement of physically counting the attached cells is cumbersome, time consuming, vulnerable to human error, and makes the assumption that all of the cells that adhered to the substrate were viable at the time of the experiment. Modifications have been made to this assay have attempted to address some of these disadvantages. For example, cells may be pre-labeled with a radioactive marker. Following the incubation and removal

of non-adherent cells, the remaining cells are lysed. The percentage of adherent cells is inferred based on the relative radioactivity of the adherent cell lysate to the initial cell suspension, with each adjusted for background counts [249]. While this approach removes the requirement of physically counting the cells, the radioactive tag is typically expensive, and hazardous. Since the radioactive material is metabolized into the cell, it does not give an accurate representation of cell-cell interactions [250]. Alternatively, cells may be labeled with a fluorescent tag, and quantified by measuring the residual fluorescence intensity of the adhered cells with a fluorescent plate reader. Presently, many commercial static adhesion assay kits are available which offer molecular markers that can be chemically tailored to target many types of cell-cell interactions. Most are based on the principle of quantifying the viable bound cells through fluorescence, or colorimetric intensity, generated through an enzymatic reaction with fluorogenic reagents or other organic chemicals which, upon undergoing an enzymatic reaction, form visibly colored by-products.

Two main classes of fluorogenic reagents are generally used: the substrates of cytosolic (cytosol is the internal fluid of the cell, where a large part of cell metabolism occurs) esterases consist of a fluorochrome group esterified with an organic acid. (Ex. Calcein acetoxymethylester (Calcein-AM) and *6* - Carboxyfluorescein diacetate (6 CDFA) [250]. The substrates for alkaline and acid phosphatase, include 2-(5'-chloro-2'- phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (CPPCQ) [251] and 5-methylumbelliferyl-phosphate (MUP) [252]. They contain a phosphate residue with an attached fluorogenic moity. The somewhat

lipophilic character of both groups of reagents allows for the selective passage of these reagents through the cell membrane of viable cells. Immediately afterward, they are hydrolyzed by intracellular enzymes, producing the hydrophilic, fluorescent reaction product. This remains within the cytoplasm and the cell now becomes detectable by measurement of a fluorescent signal that directly correlates with the cell number [253].

Colored enzymatic reactions are generated by mitochondrial dehydrogenases, acid phosphatase, hexoamidase or NADPH oxidase [253]. One of the first methods for the measurement of cell-matrix adhesion in 96 micro-well plates utilized the reduction of a tetrazolium salt (for example: 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide or MTT) by mitochondrial dehydrogenases to colored formazan products [254]. MTT is converted into a blue reaction product which can be spectrometrically quantified by absorbance at 550 nm. The formazan product however, is insoluble and requires extraction prior to measurement. The colorimetric determination of cell-matrix adhesion can also be made through phosphatase or hexosamidase dependent reactions [253].

This approach to elucidating cellular adhesion characteristics can provide excellent insight into the relative adhesion strengths and adhesive regulatory mechanisms of cells to varied substrates, but truly quantitative information, is not possible.

Quantitative detachment methods can be classified according to the type of force applied. Several categories exist: micromanipulation, centrifugation, and hydrodynamic shear force [248]. Micromanipulation quantifies adhesive forces

between a single cell, attached to a micropipette, and a (functionalized) surface or another cell. After a period of incubation, the cell is detached by pulling away the micropipette with a known applied force [255]. The forces applied with this method result in high local stresses, which are difficult to quantify, and are not necessarily representative of physiological environments, where cells are more often exposed to shear than normal forces [248]. Atomic Force Microscopy (AFM) is also used for characterizing the adhesive interactions of a single cell. This technique can provide very sensitive real-time force displacement measurements, but requires specialized, calibrated equipment, is a skill and time intensive technique [256]. AFM is well suited, however, to protein engineering, i.e. making dynamic measurements, including adhesive interactions and configurational changes, at the single molecule level [257].

Centrifugation adhesion assays are simpler by comparison, require only standard laboratory equipment (centrifuge) and allow the study of large cell populations. This assay was initially described in 1981[258]. A substrate containing adherent cells is spun at a known speed, applying controlled detachment forces ($<10^{-3}$ dynes/cell) perpendicular to the cell adhesive area [256]. Only a single force can be applied per experiment, thus multiple runs at different speeds are needed to obtain mean adhesion strength values, typically defined as the centrifugal force that results in 50% detachment [256]. This assay has been applied to the study of mechanisms of implantation using the BeWo (cytotrophoblast) cell line, or freshly isolated murine blastocysts, co-cultured with the RL95 endometrial epithelial cell line (EEC) [259].

Effects of Ca^{2++} inhibitors as well as serum in co-culture on embryo attachment, were studied, and the authors put forth that the results supported this assay as an "efficient measure of embryonic attachment under various experimental conditions"[259]. While the micromanipulation and centrifugal adhesion assays can provide quantitative information of a cells' adhesive qualities, in-vitro simulations in a dynamic environment are not possible. For example, the process of leukocyte adhesion to the endothelial cell wall is highly influenced by its presence in an environment of hydrodynamic shear. Furthermore, the parallels which have been noted between this process and that of human blastocyst implantation [142] have introduced the possibility that the initial steps in implantation also include L-selectin-mediated "tethering" (referred to by Red-Horse et al. as low strength primary bonding) of the blastocyst to the wall of the uterus [260]. Thus, static cellular adhesion assays, alone, can neither provide the insight required to completely elucidate all mechanisms which influence the adhesion process, nor can it provide quantitative information on mechanical adhesion strengths.

The differences between cellular adhesive phenomena in a dynamic versus a static environment have long been recognized, and adhesion studies which incorporate flow (hydrodynamic shear) have been reported since the mid 1900's [261]. Today, several designs exist for the quantification of cellular adhesiveness through the use of hydrodynamic flow to produce shear (detachment) forces. These include the parallel disc viscometer [262], radial flow chambers [263], the parallel plate flow apparatus [264], and the spinning disc detachment apparatus [248].

Early designs for dynamic cellular adhesion measurements were adapted for use in a biological system from instruments developed for mechanical engineering applications, such as the parallel disc viscometer [262]. This instrument consisted of two parallel discs immersed in a fluid of known viscosity (η, poise), and separated by a known distance, (*h*, cm). From fundamental rheological considerations, Weiss modeled the forces involved in the attachment of an erythrocyte to a plasma clot. He then mathematically modeled the hydrodynamic shear forces that could be applied with the parallel disc viscometer. When the upper disc rotates with a linear velocity, V cm/sec, over a circular strip of width *dr* cm, and radius *r* cm, the force acting on the strip is given by:

$$(V)(\eta)(2\pi r)(dr/h) \text{ dynes.} \quad (1)$$

Furthermore, if the angular velocity, ω, is defined by:

$$V/r = \omega \quad (2)$$

Then the shear stress is given by :

$$\frac{(\eta)\,(\omega)\,(r)}{h} \text{ dynes/cm}^2 \quad (3)$$

Weiss hypothesized that if the bottom disc had cell uniformly dispersed over its surface, then, when the upper disc rotated, "a distractive force would be applied to the adherent cell which is directly proportional to the distance of that cell from the centre of the disc". [262]. Thus the force would be at a maximum at the peripheral edge of the bottom plate, and at a minimum in the center of the plate. Weiss used this

apparatus to demonstrate the temporal changes in adhesion strength of fibroblasts to a glass substrate.

More recently, a spinning disc cell detachment apparatus has been developed and validated by Garcia, which is based on the same design principle, and consists of a single bottom plate [248]. The shear forces are generated by the chamber fluid as it undergoes laminar flow, in a radial pattern over the disc. As the disc rotates, the fluid is drawn in axially from the surroundings, and as it approaches the surface of the disc, it acquires a rotational motion which forces it to exit radially [248].

In this design, the shear stress, τ, varies linearly with radial distance and is given by:

$$\tau = 0.800\ r\ (\rho\mu\omega^3)^{1/2} \qquad (4)$$

Where ρ = fluid density, μ = fluid viscosity and ω = angular velocity.

The instrument design and flow pattern [265] are shown in figure 12.

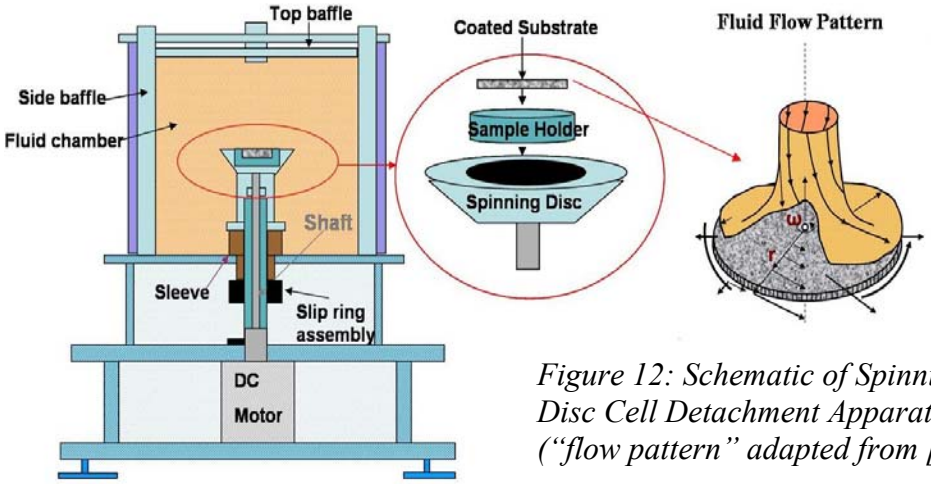


Figure 12: Schematic of Spinning Disc Cell Detachment Apparatus ("flow pattern" adapted from [265])

For a given rotational speed, the velocity and temperature of the fluid as well as the boundary layer thickness, δ, are constant, when the radius of the fluid chamber, R, is infinite compared to the radius of the spinning disc, r. The infinite disc approximation is valid when $R/\delta > 50$. This design, and in particular the fluid flow pattern, has been validated for rotational speeds of 500 to 2500 RPM through an electrochemical technique known as current-voltage (CV) scanning and the ferric/ferrocyanide redox couple. As the DC voltage in the system is varied, the transport of ions to the spinning disc was shown to be a diffusion/convection dependant process, with the resultant limiting current shown to be proportional to the rotational speed of the disc.

When cells are seeded uniformly on the disc, they will undergo detachment forces which are proportional to the radial distance from the center of the disc. The value for 50% detachment represents the average detachment shear stress for the cell population, and is used as the measure for detachment strength.[248].

Typical hydrodynamic shear detachment stresses that can be accurately generated with the (validated) spinning disc cell detachment apparatus (CDA) range from ~15 to 350 dynes/cm^2. The adhesion strengths associated with integrin-ligand interactions have been found to be in this range, and several studies have used this technique to quantify this adhesion system [248, 266-269]. Other reported applications of the spinning disc CDA include: red cell adhesion to Teflon®, glass and polyethylene [270], adhesion of 3T3 cells (immortalized fibroblast cell line from

Swiss mouse embryo tissue) to a chemically homologous series of copolymers based on hydroxyethylmethacrylate (HEMA) and ethylmethacrylate (EMA) [265].

The radial flow chamber (RFC) is another dynamic cell detachment technique which can generate a spatially dependant range of shear stresses simultaneously. Contrary to the spinning disc design, radial flow chambers utilize axisymmetric radial flow to produce a shear stress that *decreases* with *increasing radial position* [271]. The fluid is injected through a central port (usually from the bottom) into a chamber consisting of a transparent plate coated with the substrate of interest, and with cells attached. The fluid flows radially outward within a small gap, and the shear forces generated from the flow are exerted on the attached cells. A typical RFC design is shown in figure 13 [272].

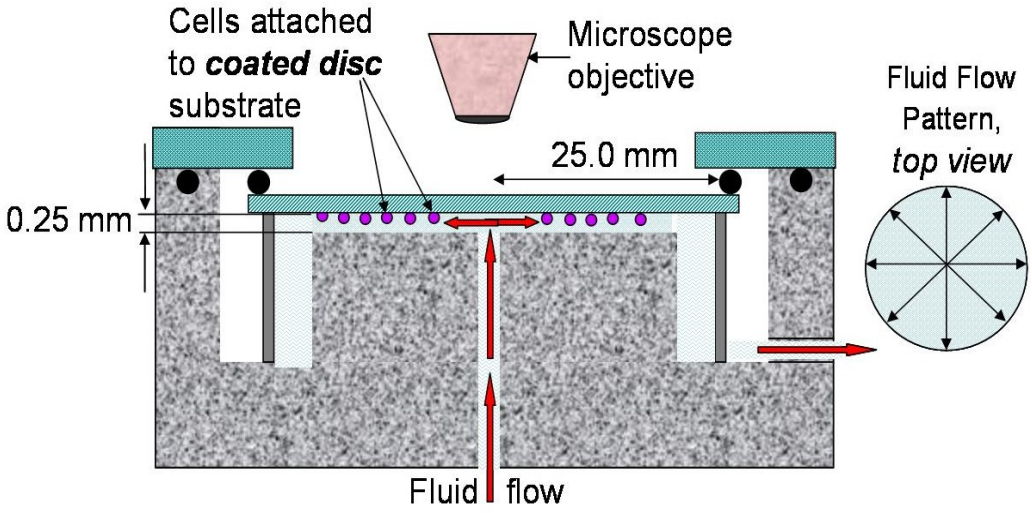


Figure 13: Typical design of a Radial Flow Chamber (Adapted from [272])

RFC adhesion assays yield a critical radius from which the adhesive force can be simply determined [273]. The shear stress, S, at any radius is given by:

$$S = \frac{3Q\mu}{\pi h^2} \quad (5)$$

where Q is the volumetric flow rate, μ is the fluid viscosity, and *h* is the gap in which the fluid is flowing. The critical shear stress, S_c, (detachment strength) is determined from the radius where the cells just begin to remain attached [271]. However, to account for the inherent heterogeneity in adhesion strengths among the attached cell populations, some researchers have defined the critical shear stress for detachment, τ_{wc}, as the wall stress at which 50% of the adherent cells detach [274-276]. This design offers the advantage of microscopic observation of the detachment events in real time. One disadvantage with this system, however, is the complexity of the hydrodynamic detachment forces at relatively small radial distances from the inlet. The assumption of "creeping flow" (a condition where the Reynolds number is very small, less than 1, and inertia effects can be ignored in comparison to the viscous resistance) is used to model the generation of detachment forces under laminar flow in this system and at this location, this assumption is not valid [277]. The range of shear detachment forces that can be generated is similar to the spinning disc CDA, ~10 to 250 dynes/cm^2 [275, 276]. As such, it has been used extensively to characterize cell detachment forces related to integrin-ligand adhesion processes [276, 278, 279].

The parallel plate flow cell detachment apparatus has a defined flow profile that closely approximates the hydrodynamic features found in the vasculature. As such, it is often the cell adhesion assay of choice in immunological studies, in particular when simulating inflammatory ailments where leukocytes adhere to endothelium under flow conditions. [280]. This apparatus was first employed for leukocyte adhesion studies by Laurence over twenty years ago [164]. This apparatus is ideally suited for observing and quantifying the process of leukocyte rolling, tethering and attachment due to the very low attachment forces which it can generate. Studies with functionalized surfaces with induced shear stresses ranging from 0.3 to 35 dynes/cm^2 are the most common [166, 281, 282]. Microscopic experimental observations from the top-down, or sometimes, from a side view in real-time are possible, and often employed. However, one disadvantage of this measurement technique, is that it allows measurements at only one shear force per experiment. The design and laminar flow pattern of a typical parallel plate flow apparatus is shown in figure 14.

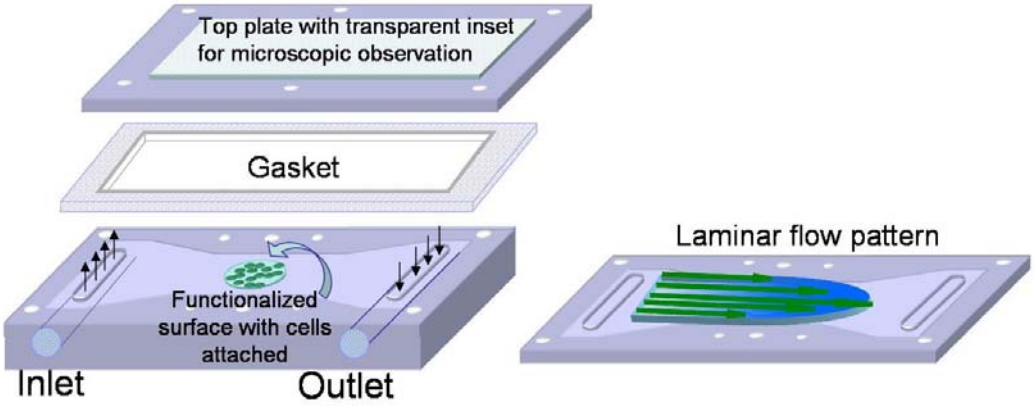


Figure 14: Schematic of a Parallel Plate Flow Chamber, showing laminar flow pattern

The configuration shown in figure 13 shows a functionalized bottom surface with cells attached, which will be detached under hydrodynamic shear, thus defining a cell *detachment* assay. In another variation of this assay, the cells are perfused in the chamber with the fluid, and their rolling and/or subsequent attachment behavior can be observed and quantified, thus defining a cell *attachment* assay. In both cases, either the bottom or the top plate may be functionalized for the adhesion assay. Laminar flow conditions are predicted when the Reynolds number < 2. (In fluid mechanics, this is the ratio of inertial forces to viscous forces). The fluid velocity profile, as shown in figure 11 is parabolic. The wall shear stress, τ, can be calculated from the momentum balance for a Newtonian fluid [283], and is given by:

$$\tau = \frac{3\mu Q}{2a^2 b} \qquad (6)$$

where μ= Coefficient of viscosity, Q = volumetric flow rate, 2a = channel height and b = channel width [164].

The process of leukocyte adhesion has been well characterized with the parallel plate flow apparatus. The surface expression of adhesion molecules such as ICAM-1 [284], VCAM-1 [285], E-Selectin [286], P-Selectin [287], L-selectin and PNAd [288], $\alpha_4\beta_1$ [289] and PSGL-1 [290] and other L-selectin ligands expressing sialyl Lewis *x* epitope [291] have all been characterized in this manner. Neutrophils have been shown to attach to L-selectin substrates with strengths of 1-3 dynes/cm^2 [166]. Studies in parallel plate flow chambers have allowed the observation and

quantification of the time constant of "rachet-like" steps involved in leukocyte rolling and tethering on selectin-ligand substrates as well as the estimation of a single bond force of 125 pN (pico-Newton) or below for all selectin-ligand bonds[288]. Under physiologic flow conditions, L-selectin in the neutrophil membrane has been demonstrated to redistribute, forming a "cap" or polarity of expression in the cells [171]. This interesting phenomenon was induced and characterized in a parallel plate flow chamber.

Characterization of trophoblast cellular adhesive function or protein expression in a flow environment, with a focus on implantation events, has not been reported. However, one study has been reported using term human placental cytotrophoblast cells (HuPla), alone and in co-culture with uterine endothelial cells [292]. The objective of this work was to elucidate some of the migratory aspects of trophoblast cells in the process of homing to the maternal vasculature after implantation, under conditions of shear stress, ranging from 1 to 30 dynes/cm^2. The parallel plate flow chamber was a critical tool in the discovery that the ability of trophoblasts to migrate against flow at physiological levels of shear stress is dependent on the presence of endothelial cells indicating that factors expressed on both trophoblast and endothelial cells play a role in this process [292]. For similar reasons, it is felt that this apparatus could also play a critical in understanding trophoblast/uterine epithelial interactions under conditions of hydrodynamic shear stress during implantation.

CHAPTER 2. HYPOTHESIS AND OBJECTIVE

The ultimate objective of this thesis is to understand the biochemical and mechanical mechanisms responsible for the initial events which lead to the successful attachment of the blastocyst to the uterine epithelium, for pregnancy. From this insight, we would endeavor to develop clinically relevant treatment therapies which could affect the necessary conditions to support the formation of human life. One of the biochemical mechanisms that has recently been identified as important in the initial attachment process is the L-selectin/L-selectin ligand adhesion system [141]. Furthermore, based on what is known about the protein adhesion molecules involved in the implantation process, it has been noted that strong similarities with the leukocyte attachment and extravasation process, exist [142], whereby L-selectin/L-selectin ligands interact to form a weak bond *primary* between the rolling leukocyte and the vascular endothelium, which results in the capture of the leukocyte. From this interaction, the integrin/ligand system is thought to be activated, mediating the formation of stronger bonds through the integrin/ligand adhesion system. Yet, there is still very little understood about this process. In particular, it is not known how the hormonal environment mediates the expression of the cell adhesion molecules nor how the mechanical aspects of this process, both quantitative and qualitative, affect the outcome. Our overall hypothesis is that the expression and retention of a *critical* level of L-selectin and L-selectin ligands are necessary to facilitate adequate initial attachment of the blastocyst to the uterine epithelium. We specifically hypothesize the following:

- That the L-selectin adhesion system is modulated by the local environment of steroid hormones, cytokines and inflammatory mediators *as well as* mechanical shear forces due to flow.
- That following the L-selectin mediated (low strength) attachment process, the trophectoderm will activate RDG containing adhesion proteins, such as fibronectin, vitronectin and fibrinogen which will be responsible for interacting with the integrins expressed by the uterine epithelial cells providing a higher strength secondary attachment.
- That the attachment strengths in this system will be comparable to those reported for the leukocyte-vascular endothelial system.

The objective of this project is to understand, and quantify where possible, the biochemical and mechanical mechanisms responsible for blastocyst attachment to the uterine epithelium, through the use of an optimized cell model, of established trophoblast-like and uterine epithelial cell lines. This will be accomplished through the following specific steps:

a) The basal and temporal expression of the L-selectin adhesion system, in the presence of steroid hormones, will be quantified through Western Blot analysis, and flow cytometry. The specification of the optimized cell model will be based on appropriate responses of up or down regulation of L-selectin in the presence of <u>normal</u> hormonal influences.
b) The optimized cell model will be characterized for L-selectin modulation and shedding in response to therapeutic hormones and fluid flow, with the use of

Western Blot analysis, and ELISA. This will provide insight into the regulation of L-selectin in the presence of shear flow forces, as well as the effect of therapeutic hormones, which are used frequently in IVF protocol, on L-selectin expression.

c) Systems for the measurement of the temporal attachment strengths of primary and secondary adhesion systems will be designed, constructed and validated. A parallel plate flow apparatus will be employed to quantify low strength primary attachments, and a spinning disc cell detachment apparatus will be used to measure more robust secondary interactions between the Ishikawa uterine epithelial cell line and substrates which mimic the trophectoderm.

CHAPTER 3: ESTABLISHING A CELL MODEL

3.1 Introduction

Embryo implantation in mammals varies somewhat with the species, with human implantation events being unique in many ways. Yet it is not possible, for a number of practical and ethical reasons, to study directly the physiology of implantation in statistically large numbers of human subjects. Therefore, animal and cell-based models are a necessary factor in unraveling the complexities of the molecular and mechanical events involved in human blastocyst implantation. The animal models which are commonly used for such studies are: mice, rats, guinea pigs, pigs, rabbits, sheep, cows and primates such as the cynomolgus macaque, the marmoset monkey and the baboon [293]. In mammals, the processes *between* fertilization and the initiation of implantation generally follows a well conserved autonomous process [294], through multiple steps of mitotic cell division until it becomes a multi-cellular ball of differentiated cells called the blastocyst. Upon shedding the zona pellucida, the blastocyst becomes implantation competent, and the diversity between the species is clearly seen at this point, even at the morphological level. In humans, implantation usually occurs between days 7-9 post fertilization, in mice, it occurs at day 4 and in cows, it does not occur until 30 days after fertilization [294, 295]. Implantation in mammals has been divided into three broad categories: centric, eccentric and interstitial [296]. With centric implantation, the embryo expands to a large size before implantation, then remains in the center of the uterus. Examples include carnivores, ruminants, horses, and pigs. In eccentric implantation the blastocyst is

small at the time of implantation, and the site of implantation is within the endometrium on the side of the uterus, usually opposite to the mesometrium. Examples include rats and mice. Interstitial implantation occurs when a small blastocyst erodes through endometrial epithelium into subepithelial connective tissue. This process is also called nidation ("nest making") and is seen in primates, and guinea pigs. Figure 15 schematically illustrates each of these processes [117].

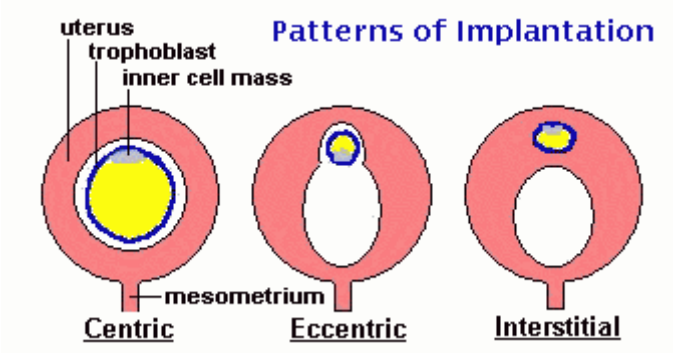


Figure 15: The three different types of implantation: interstitial implantation occurs in humans [117]

It has been established that the primary factors that stipulate endometrial receptivity in humans are the ovarian steroids, estrogen (E2), and progesterone (P4). The physiological effect of ovarian steroids in the uterus are best understood in the mouse model, where gene targeting experiments have elucidated the roles of these receptors in uterine function [297]. Biomedical research in murine models has been greatly facilitated by the use of "knock-out" mice, that is those which have been bred missing a single gene, and reproductive studies are no exception. In the case of implantation, however, the human process differs significantly and no suitable animal model has

been found. Even our closest species, the rhesus monkeys, are different (superficial implantation, limited decidualization, dual insertion at opposite poles etc. . . .). This uniqueness is perhaps best illustrated by the fact that extra-uterine pregnancies are not uncommon in humans whereas they are almost unknown in other mammals [53]. Although human blastocysts, produced in IVF programs, have undergone detailed observation by conventional and electron microscopy, neither the stage of adhesion to the uterine surface nor the penetration phase have been observed in the human. Consequently the nature of these crucial events must be deduced from in vitro models using cell models of human trophoblastic cells [53], and uterine epithelial model cell lines, or the human tissue itself.

Several human trophoblast cell lines exist and have been studied as models for various aspects of human trophoblast function in the implantation and gestation process. In order to maximize the possibilities of replicating the protein expression signature of implantation ready trophoblast cells, it is important to use a cell line that has been established from fetal tissue harvested no later than first trimester. The cell line should possess the characteristic invasive properties of normal first-trimester trophoblast cells [298]. Three human tissue derived cell lines meet these criteria: JAR [299] Jeg-3 [300] and BeWo [301]. All are *choriocarcinoma* cell lines. Interestingly, many elements of trophoblast invasion are similar to events that occur during tumor cell invasion and these similarities suggest that that the two invasive properties may share certain common mechanisms [302]. These three cell lines have been used (many times in parallel) for reproductive studies of processes occurring in

the period ranging from pre-implantation to the establishment of the fetal maternal vasculature.

JAR cells have been used to monitor adhesive forces, via atomic force microscopy (AFM) between the trophoblast and uterine epithelium [303], an aspect of cellular interactions that would provide insight into implantation mechanisms. They have been recognized for their "adhesiveness" and as such been utilized in studies with uterine epithelial cell lines (RL-95) to characterize certain signaling events during embryo implantation such as the Ca^{++} influx [304] or upregulation of RhoA [305] (a small GTPase protein known to regulate the actin cytoskeleton in the formation of stress fibers) that occurs in uterine epithelial cells as a result of trophoblast cell attachment. Interestingly, both of these studies used multicellular JAR cell "spheroids", a configuration that closely resembles a trophectoderm. They have been also characterized as a potential model for studies of transepithelial transport in the placenta (a post-implantation process), and were found to be unsuitable[306].

The hormone secreting properties of the trophoblast cell line, BeWo, have been shown to closely match those of normal trophoblast cells [307]. The levels of IL-8R mRNA and protein expression in BeWo cells is similar to those in first trimester primary trophoblast cells [308]. Epidermal growth factor (EGF) has been shown to dose-dependently stimulate the invasiveness of BeWo cells by inducing α2 integrin expression, as well as affect the upregulation of human chorionic gonadotrophin (hCG) [308]. Finally, BeWo spheroids were used in co-culture attachment studies with RL95-2 endometrial epithelial cells (EEC), and were found to cause the

upregulation of p38 MAPK (mitogen activated protein kinase) and ERK (extracellular signaling-related kinase) in the EEC's [309]. These signaling pathways are felt to be critically important in the implantation process.

Jeg-3 cells, have been utilized in several studies which focus primarily on hormonal expression and adhesion molecule modulation around the pre-implantation and initial implantation period. Jeg-3 cells, as well as normal trophoblast cell line (NPC), have been used to investigate the signaling pathway which results in the inhibitory effect of TGF-β on the production of estrogen and progesterone [114]. Jeg-3 cells have been used to elucidate effect of LIF [310] and IL-17 [311] on the invasive properties of trophoblast cells. The expression and regulation of matrix metalloproteinases (MMP's) has been found to be up-regulated in Jeg-3 cells by fibronectin through the focal adhesion kinase (FAK)/$p42^{MAPK}$ signaling pathway [280]. It was shown that cadherin-11 (a membrane protein that mediates intercellular adhesion) contributes to the morphological and functional differentiation of the Jeg-3 cells in a highly specific manner [312].

Several studies have included comparative evaluations of two or three of these cell lines. RT-PCR was used to assess the presence of mRNA for the expression of cytokines known to be involved in the regulation of critical implantation related events in JAR, Jeg-3 and BeWo cells. All were found to express IL-6, IL-10, IFN-α, IFN-β, and GM-CSF [313]. High numbers of natural killer (NK) cells which are present in the uterine mucosa during the pre-implantation period and early pregnancy have been implicated in the regulation of trophoblast invasion. Both Jeg-3 and BeWo

cells have been validated as appropriate trophoblast cell lines in a three-dimensional co culture in vitro cell model with uterine NK cells to study this process in detail [314]. Osteopontin has been shown to play an important role in embryo implantation by supporting the attachment of trophoblast cells to the uterine epithelium [315]. A recent study, using JAR, Jeg-3 and BeWo cells focused on the signaling pathways associated with osteopontin (OPN) mediated cell migration [316].

Hohn et al. showed, in a co culture model with RL95-2 cells, that the level of adhesive interactions of all three trophoblastic cell lines was inversely related to the level of differentiation of the trophoblast cells [317]. All three cell lines were pretreated with agents which are known to promote differentiation of cells such as methotrexate (MTX), dibutyryl cyclic AMP (dbcAMP) and phorbol-12-myristate-13-acetate (PMA). In addition to increased secretion of hCG (human chorionic gonadotropin), a characteristic marker of early trophoblast differentiation, they found in every case that their adhesion to the uterine epithelial cell line was significantly reduced[317]. Though molecular aspects of the adhesive process were not the focus of this study in particular, the results support the appropriateness of JAR, Jeg-3 and BeWo cells for use in examining the implantation process.

Two human uterine epithelial derived adenocarcinoma cell lines have been widely used for in-vitro studies of the early reproductive process: RL95 and Ishikawa. The RL95 cell line was established in 1983 by Way [318] and it has been used in numerous studies which focus on blastocyst-endometrial binding interactions[259, 303-305, 309, 317, 319]. RL95 cells have been extensively used to investigate the

potential function of heparin sulfate proteoglycans (HSPGs) in human implantation [319, 320]. In one study, initial binding interactions between JAR and RL95 cells was shown to be mediated by cell surface glycosaminoglycans (GAGs) with heparin-like properties, an observation consistent with an important role for HS and heparin-like GAGs as well as their corresponding binding sites in early stages of human trophoblast-uterine epithelial cell binding [319].

Both the RL95 and Ishikawa cell line have been used in the study of progesterone-dependent regulation of MUC1, a cell membrane associated polymorphic mucin [321]. This protein, expressed on the endometrial epithelium throughout the menstrual cycle, is characterized by anti-adhesive properties, and its function in the implantation process is poorly understood.

The Ishikawa cell line was established in 1985 by Nashida [322]. This cell line has been proven to be stable, hormonally responsive, and has been shown to express endometrial proteins in a hormonally responsive manner [323]. Ishikawa cells possess both estrogen and progesterone receptors, which are regulated in a manner similar to that of the human endometrium [324]. They also express many of the same enzymes and structural proteins found in the normal endometrium [325]. The integrin expression characteristics in the Ishikawa cell line have been well characterized. As with normal endometrial epithelium, they maintain constitutive expression of $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_4$, and show cycle dependent expression of $\alpha_1\beta_1$ [324] and $\alpha_v\beta_3$ [325]

The similarities between normal uterine epithelial tissue and Ishikawa cells was further validated by the demonstration of comparable epidermal growth factor (EGF,

a biomarker of uterine receptivity) regulation of $\alpha_v\beta_3$ expression [326] and the leptin mediated regulation of LIF, LIF-R and IL-1 ligand [327].

As an acceptable in vitro model for normal uterine epithelial tissue, Ishikawa cells have provided useful insight into the underlying mechanisms contributing to conditions of infertility, and the effectiveness of possible clinical treatments. The Ishikawa cell line has been used to investigate the role of androgen receptors (AR) in human endometrium. Endometrial AR have been shown to be biologically functional, inducing prolactin secretion in stromal cells *in vitro* [328] but elevated endometrial AR expression in women with poly cystic ovarian syndrome (PCOS) has been indicated as a contributing factor in poor reproductive performance of women with PCOS [131]. The up-regulation of AR in response to estrogen, and down regulation of AR by progestins in Ishikawa cells has been demonstrated [323]. Androgens were found to down regulate the expression of $\alpha_v\beta_3$ in Ishikawa cells, suggesting possibly one of many potential alterations in endometrial epithelial protein expression that may occur in response to elevated androgens in women with PCOS [131].

One of the specific goals of this work is to establish and validate a cell model for studying the L-selectin/L-selectin ligand adhesion system using a human trophoblast derived cell line and a uterine epithelial cell line. Of the many implantation related studies using the three trophoblast cell lines, no one cell line emerges as an ideal candidate, so all three cell lines will be evaluated for normal hormonal regulation of L-selectin expression. Conversely, since Ishikawa cells have been particularly well characterized with regard to the expression of protein biomarkers of uterine

receptivity, they are well suited for studies focused on the L-selectin mediated mechanisms of blastocyst implantation in humans, and will therefore be the uterine epithelial cell model of choice.

3.2 Materials and Methods

Cell Lines and Cell Culture

JAR, Jeg-3, and BeWo trophoblast derived choriocarcinoma cell lines were obtained from American Type Culture Collection (*ATCC, Rockland Md*.). The U-937 cell line, a non adherent monocytic leukemia cell line which has been shown to constitutively express L-selectin (deCoupade, 2003), was a generous gift from Dr. Monika Jost, (Drexel University College of Medicine). Ishikawa cells were purchased from European Collection of Cell Cultures (*ECACC, Wiltshire, UK*). Cell lysates from the drosophila cell line, Deg-5r, (a generous gift from Dr. Noreen Robertson, Drexel University College of Medicine) were used as a negative control for L-selectin and L-selectin ligands. All cell lines, except for Deg-5r, were routinely cultured at 37^{o}C, in a humid atmosphere with 5% CO_2. U-937 and JAR cells were maintained in RPMI-1640 culture medium (*ATCC # 30-2001*), BeWo Cells were maintained in ATCC formulated F-12K culture medium (*ATCC # 30-2004*) and Jeg-3 and Ishikawa cells were maintained in Eagle's Minimum Essential Medium (MEM) purchased from ATCC *(#30-2003*). All culture media was supplemented with 10% Fetal Calf Serum (*Fisher Scientific*). All cells were routinely passaged every 5-7 days. For adherent cells (JAR, Jeg-3, BeWo and Ishikawa) the culture medium was first removed, and cells were washed once with trypsin/EDTA (*Cellgro*) to remove all traces of serum

which contains trypsin inhibitor. Cells were then incubated at 37^{o}C in fresh trypsin for 5-15 minutes, until the cell layer was dispersed. FCS supplemented culture medium was then added to the flask to inhibit the trypsin, and the cell suspension was then centrifuged, resuspended in fresh medium and seeded into new culture flasks. Non-adherent cells (U 937) were passaged by simply aliquoting a predetermined portion (10-15%) of the cell suspension into a new flask and supplementing with fresh culture medium A detailed protocol for cell passaging can be found in Appendix A.

Hormonal Conditioning

Cells were first passaged and incubated for 24h under normal conditions in order to assure log-growth properties. Cells were then adapted for 24h in phenol red free culture medium: RPMI-1640 (*Fisher Scientific #MT-17-105CV*) for the U 937 and JAR cells, and D-MEM F-12 (*Gibco/Invitrogen #21041)* supplemented with 10% charcoal stripped FBS to eliminate background stimulation by estrogen-mimetics or FBS-derived hormones. Cells were treated with physiological concentrations of relevant hormones (10^{-8} M estradiol, 10^{-6} M progesterone), or a combination of both for 24h. A detailed protocol for hormonal conditioning of the cells can be found in Appendix B. Cells were then harvested and processed into cell lysates for analysis by Western Blotting.

Cell Lysate Preparation

For the preparation of cell lysates, a modified form of a lysis buffer previously described [120] was used: Into molecular grade water was placed 10 mM Tris-

Acetate @ pH 8.0, 5 mM CaCl , 0.1% NP-40, 1 mM Sodium Orthovanadate, 0.01 mM NaF, 0.10 mg/ml Pepstatin, 1 "Complete Mini" tablet (*Roche*, *Nutley, NJ*), 0.5 uM Chymostatin, and 10 ug/ml Benzamidine HCl. All lysates were prepared at 4^{o}C. Cell cultures were washed 1X in cold PBS, followed by a 5 minute incubation in lysis buffer. they were then removed from the culture flask by scraping, and incubated on ice for 30 minutes with regular agitation. Cells were lysed, centrifuged to concentrate the cellular debris, and the lysate solution was removed. Total cellular protein content was determined by a bicinchoninic acid (BCA) protein assay (*Pierce, Rockford, IL*). Lysates were prepared for Western Blotting by mixing the lysate in a 1:1 ratio with Laemmli sample buffer (*Bio-Rad, Hercules, CA*), with β-mercaptoethanol added and boiling for 5 minutes. A detailed protocol for cell lysate preparation can be found in Appendix C.

Antibodies and Antigens

Recombitant human L-selectin FC chimera was obtained from *R & D Systems (San Diego, CA*). In addition to the U 937 cells, this antigen was used as a positive control for the detection of L-selectin. The primary antibodies used for Western Blot experiments were: LEAF purified anti-human CD62L (L-selectin) (DREG-56) which was obtained from *BioLegend* (*San Diego, CA*), MECA-79 rat anti-mouse monoclonal antibody which was obtained from *BD Pharmingen* (*San Jose, CA)*, mouse anti-human mucosal addressin cell adhesion molecule 1 (MAdCAM-1) which was obtained from *Serotec, Raleigh, NC (# MCA2096Z and MCA23)*. β-Actin rabbit polyclonal antibody was obtained from *Cell Signaling Technology, Inc*. *(Danvers,*

MA). The quantification of actin in the cell lysates provided the internal loading control. The primary antibody for L-selectin detection used in flow cytometry was anti-human L-selectin (CD62L) monoclonal antibody #BBA24 *(R&D Systems, Minneapolis, MN).*

Isotype controls were used in immunohistochemistry and flow cytometry experiments. Affinity purified rat IgM isotype control antibody was obtained from *eBioscience* (San Diego, CA). Affinity purified mouse IgG_1 Isotype control *eBioscience* (San Diego, CA). Secondary antibodies are used after incubation of the primary antibody to couple with the species-specific portion of the primary antibody and provide the chemically reactive label that allows direct detection. The secondary antibodies used in Western Blots were: peroxidase-conjugated AffiniPure goat anti-rat IgM which was obtained from *Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA)* HRP goat anti-mouse IgG polyclonal antibody which was obtained from *BioLegend* (San Diego, CA) and anti-rabbit IgG HRP-linked antibody which was obtained from *Cell Signaling Technology, Inc. (Danvers, MA).* The secondary antibody used for L-selectin detection in flow cytometry was Alexa-flor 488 goat anti-mouse IgG_1 # A11001 *(Molecular Probes/ Invitrogen, Carlsbad, CA).* At times, an intermediate antibody system is used to enhance the sensitivity of the detection and this was the case in the immunohistochemistry experiments. Typically, this is a streptavidin/biotin coupling system. A Streptavidin/Biotin blocking Kit, #SP 2002, was obtained from Vector Labs. (*Burlingame, CA*). The biotinylated secondary antibodies used in the immunohistochemistry protocol were: Biotinylated anti-mouse

IgG #BA-9200, Biotinylated anti-rabbit IgG # BA-1000 (both from *Vector Labs*) and Biotin Conjugated Antibody to Rat IgM #16-16-03 (KPL, Gaithersburg, MD). Alkaline phosphatase Strep-Avidin, #SA-5100 and the BCIP/ NPT reagent kit, #SK-5400, were also obtained from Vector Labs.

Western Blotting

Sample lysates were run on 10 well 4-20% SDS-PAGE mini-gels (*Bio-Rad, #161-1105)* under reducing conditions. Each well was loaded with the same amount of total protein. The protein mixture was electrophoretically separated by molecular weight at 100V for 1.5 hours. The separated proteins were then transferred to nitrocellulose membranes at 100V for 1 hour. The non-specific binding sites on the nitrocellulose membranes were blocked with 7% powdered milk in PBS plus .1% Tween-20 (PBS-T) for 1hour at room temperature. The membranes were then incubated with the primary antibody in a solution of 5% powdered milk in PBS-T overnight at 4°C. Membranes were washed in PBS-T, then incubated with the Horseradish Peroxidase (HRP) conjugated secondary antibody in a solution of 5% powdered milk in PBS-T for 1 hour with agitation. A final washing step in PBS-T was performed before incubating the membranes in ECL chemiluminescent reagent (*Amersham*, *Arlington Heights, IL*) and developing on x-ray film. A detailed protocol for western blotting can be found in Appendix D.

Immunohistochemistry

Ishikawa cells were grown for 24 hrs. on 4-well Permanox chamber slides *(Fisher Scientific #12-565-21)*. All of the following steps were done at room temperature,

unless otherwise noted. Cells were fixed with 4% paraformaldehyde for 30 min. This was followed by washing in PBS, a 1h blocking step in 10% normal goat serum (NGS), and a PBS with 0.05% Tween 20 (PBS-T) wash. Cells were blocked with streptavidin D for 15 minutes, followed by a PBS-T wash and a 15 minute block with biotin solution. Each chamber was incubated with a selected primary antibody, (anti-actin, MECA-79 (for L-selectin ligands) or DREG-56 (for L-selectin) in 5% NGS in PBS-T, at 4^{o}C overnight. The cells were then washed in PBS-T, followed by a 1.5h incubation with the corresponding biotinylated secondary antibody. After washing in PBS-T, the samples were incubated for 1.5h with alkaline phosphatase Strep-Avidin diluted in 10% NGS in PBS-T, followed by two washes in PBS-T and a final wash in PBS. An alkaline phosphatase system was used for visualization. BCIP/ NPT/levamisole reagents (*Vector Labs*) were prepared and used according to the instructions. This substrate forms a blue reaction product which can be easily observed microscopically. Slides were washed in DI water air dried and preserved with coverslips and aqueous mounting media for future analysis. A detailed protocol for immunohistochemistry of Ishikawa cells can be found in Appendix E.

Flow Cytometry

Adherent cells were detached by trypsinization, neutralized with fetal calf serum and washed in PBS. For staining, cells were resuspended in staining buffer (PBS with 0.2% BSA) and incubated with either L-selectin primary antibody (clone 4G8), or isotype-matched control (clone MOPC-1, *Beckman-Coulter, Fullerton, CA)*, for 20 minutes at room temperature, followed by incubation with Alexa-Fluor 488

conjugated goat-anti mouse secondary antibody, (20ug/ml) in staining buffer for 15 minutes at room temperature. Between antibody incubations, cells were washed with washing buffer (3% (v/v) FCS in PBS. Flow cytometry analysis was performed using a FACSORT flow cytometer (*BD Biosciences, San Diego, CA*) with 488 nm excitation from an argon ion laser at 15 mW. Forward scatter threshold was set to exclude small debris. Alexa Fluor 488 log fluorescence was captured on the FL1 channel equipped with a 530nm wavelength filter with 30nm bandwidth. Data acquisition was done using Lysis II software (version 2.0, *BD Biosciences, San Jose, CA*). At least ten thousand events were acquired per sample. Data analysis was performed using WinMDI software (Joseph Trotter, *The Scripps Research Institute, La Jolla, CA*, available online from http://facs.scripps.edu).

Statistical Analysis

Data were typically calculated as mean values with standard error of the mean from several experiments, performed on different days, with an n of ≥3, unless otherwise noted. Additionally, the significance of the differences was analyzed using Student's two tailed *t*-test, comparing experimental groups with controls. Limits of confidence are indicated for the 95% level, that is, error probability will be $P \leq 0.05$ when the limits of confidence do not overlap.

3.3 Results

Western Blot Detection of L-selectin Expression

In figure 16 representative samples of a typical western blot for each cell type, showing the L-selectin expression of the cell line with the hormonal treatment, and

corresponding actin bands. Cellular L-selectin is expressed at ~70 kDa; The L-selectin positive control, recombitant human L-selectin/Fc Chimera, consists of the extracellular domain of human L-selectin (amino acid residues 1 - 332), fused to the carboxy-terminal 6X histidine-tagged Fc region of human IgG_1 via a polypeptide linker. The chimeric protein was expressed in a mouse myeloma cell line, NS0. As a result of glycosylation, this protein is specified by the manufacturer to migrate under reducing conditions in SDS PAGE at 90-100 kDa.(R&D Systems, 2007) Actin is expressed at 40 kDa. For the negative control, only the actin band is seen. The blots are arranged in 3 sample sets, with each sample set consisting of 1 lysate of treated cells, and 1 lysate of non-treated cells.

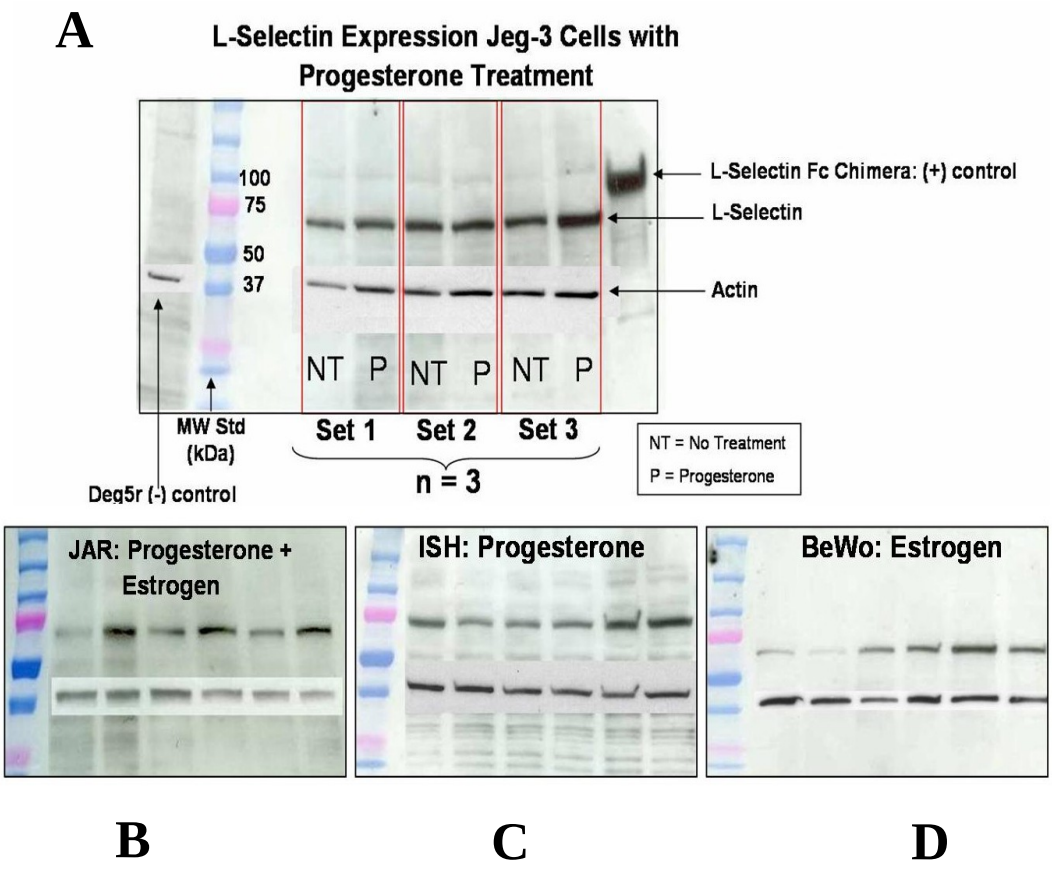


Figure 16: Representative western blots for each cell line, with hormone treatment. In each blot, a negative control, a positive control and a molecular weight standard was run in conjunction with the lysate sets (one control, and one treated) prepared from three duplicate runs for each cell line and each treatment.

A) Western Blot, fully labeled, showing bands for Jeg-3 cell lysates, where the treated cells were conditioned in progesterone for 24 hours.

B) Western Blot showing bands for JAR cell lysates where the treated cells were conditioned in Progesterone + Estrogen for 24 hours. The six bands are divided into three sets, as shown in A.

C) Western Blot showing bands for Ishikawa cell lysates where the treated cells were conditioned in Progesterone for 24 hours. The six bands are divided into three sets, as shown in A.

D) Western Blot showing bands for BeWo cell lysates where the treated cells were conditioned in Estrogen for 24 hours. The six bands are divided into three sets, as shown in A.

For Western Blot analysis, all results are presented as *relative protein expression*. First, the intensity of all protein bands was quantified with the Kodak "1D" image analyzer and associated software.

The *Standardized Intensity* (SI) of the protein band was calculated as the ratio of total protein loading, as indicated by the *intensity* of the corresponding actin band (I_{ACTIN}), to the *intensity* of the corresponding protein band ($I_{TREATED}$ or $I_{CONTROL}$). Thus:

$$\frac{I_{TREATED}}{I_{ACTIN}} = SI_{TREATED,} \quad \text{and} \quad \frac{I_{CONTROL}}{I_{ACTIN}} = SI_{CONTROL}$$

The *Normalized Protein Expression* of the cells was calculated as the ratio of the standardized intensity of the treated cells versus the standardized intensity of the controls:

$$\frac{SI_{TREATED}}{SI_{CONTROL}} = \textbf{Normalized Expression}$$

Thus the normalized expression of the control samples is always "1".

Figure 17 shows the modulation of L-selectin expression on all trophoblast cell lines (JAR, Jeg-3, BeWo) as well as the uterine epithelial cell line (Ishikawa).

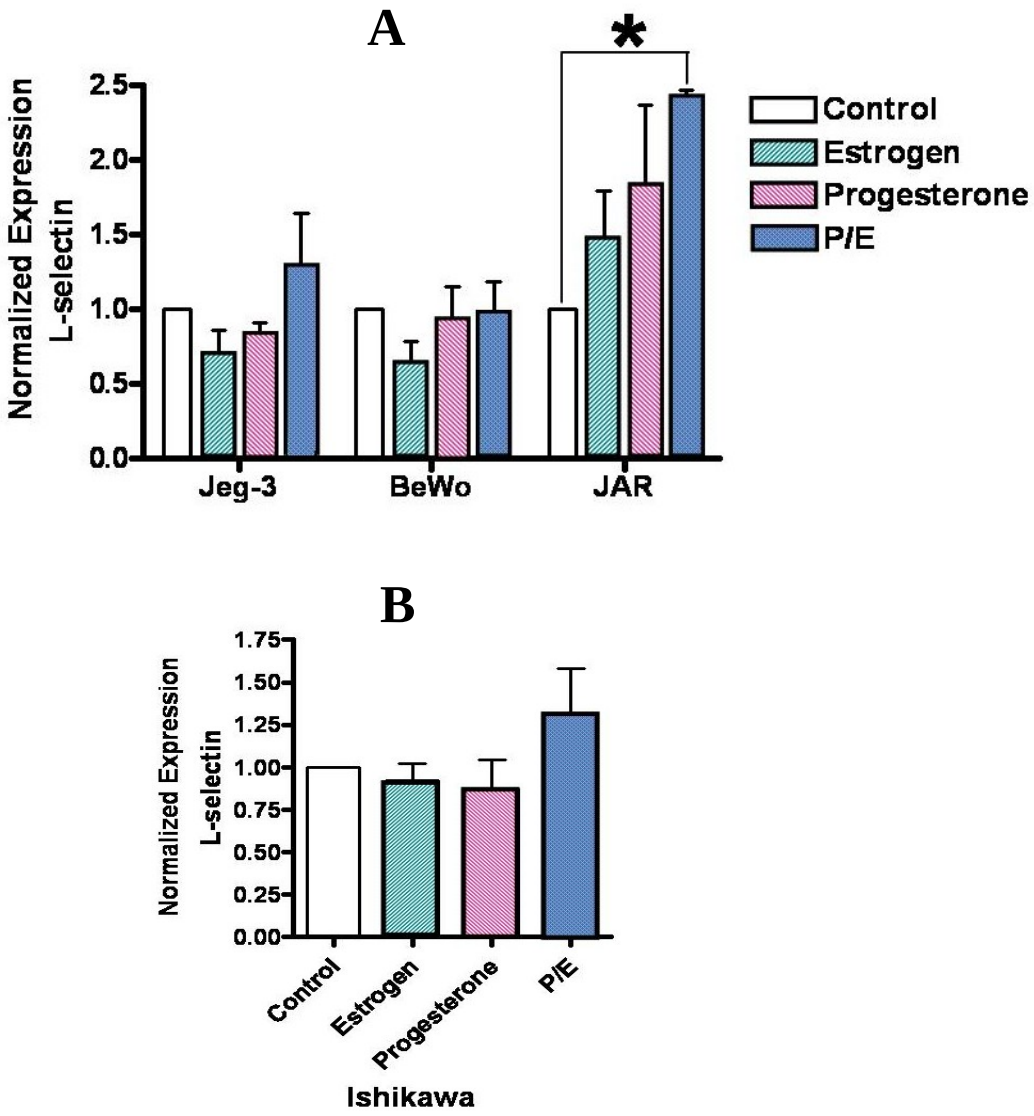


Figure 17: Normalized Expression of L-selectin in Trophoblast Cell lines (A) and Uterine Epithelial Cell Line (B)

Detection of L-selectin Expression through Flow Cytometry:

In figure 18, the basal expression of L-selectin, as detected through flow cytometry, is shown for all trophoblast cell lines, as well as the monocytic leukemia cell line, U-937, which was used as a positive control.

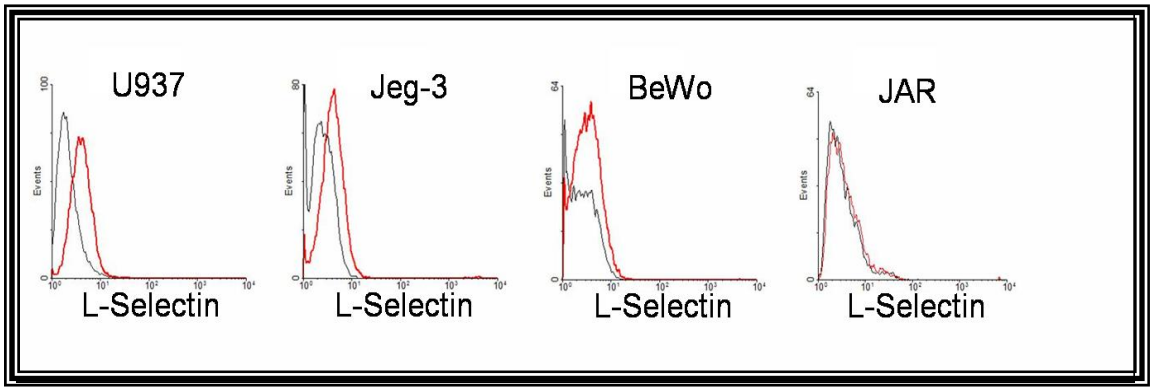


Figure 18: Determination of basal expression of L-selectin in all three trophoblast cell lines by flow cytometry

Detection of L-selectin Ligands

The uterine epithelial derived Ishikawa cell line was probed for expression of L-selectin ligands, at a basal level and under hormonal stimulation. Three antibodies (MECA-79, and two anti-MadCAM-1) were used in this work. Figure 19 shows a representative western blot in which MECA-79 antibody was used to detect the important *class* of L-selectin ligands which contain sulfate and carbohydrate epitopes. Prominent bands were seen at 90, 80, 50 and 40 kDa. Since one of the bands evaluated in the MECA-79 blot was at 40 kDa, the actin bands are shown separately.

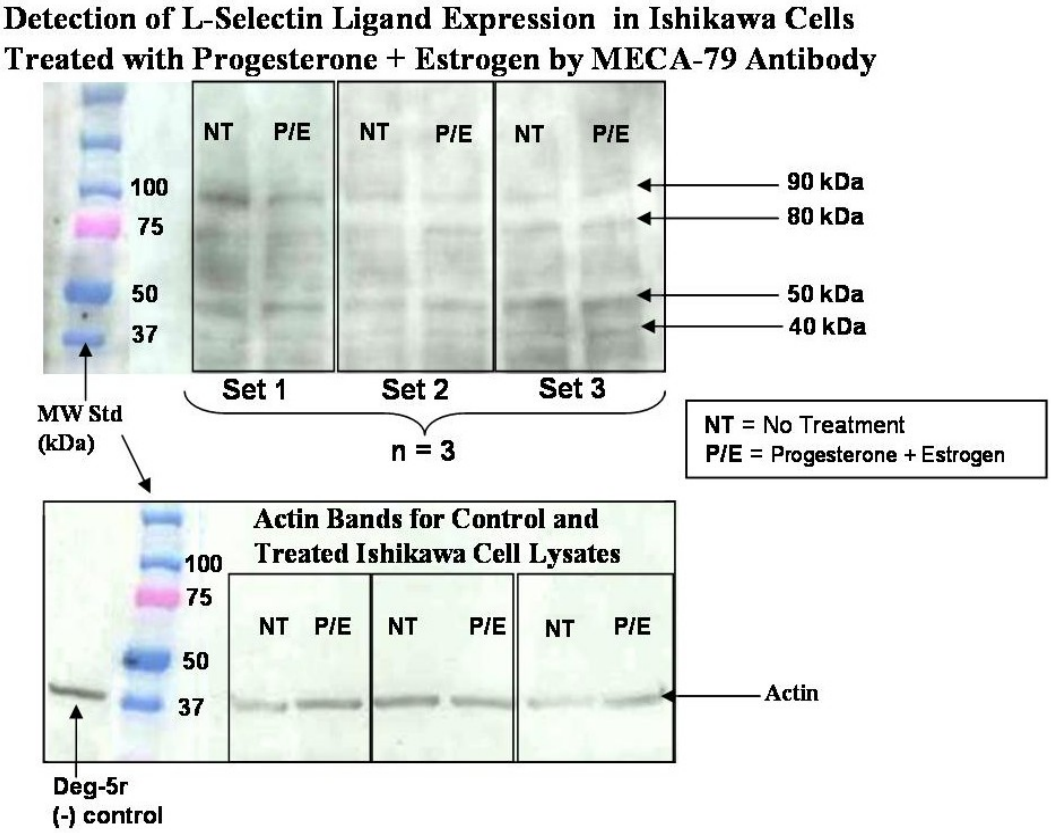


Figure 19: Representative western blot for L-selectin Ligand detection in Ishikawa cells with MECA-79 antibody.

The expression of the MECA-79 associated L-selectin ligands was analyzed by the same procedure presented previously (*3.3 Detection of L-selectin*). The most prominent bands occurred at 90, 80, 50 and 40 kDa, and these, along with a "total intensity" reading were used to calculate the relative expression of L-selectin ligands. The results are shown in figure 20.

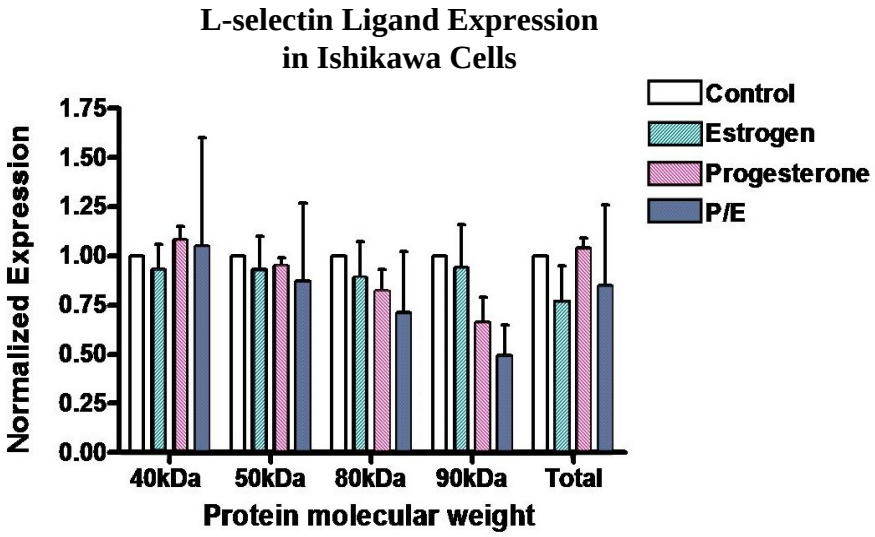


Figure 20: Normalized expression of L-selectin ligands, containing the sialyl Lewis x epitope, as detected by MECA-79 antibody.

Figure 20 shows the results of immunohistochemical analysis of Ishikawa cells for MECA-79 reactivity.

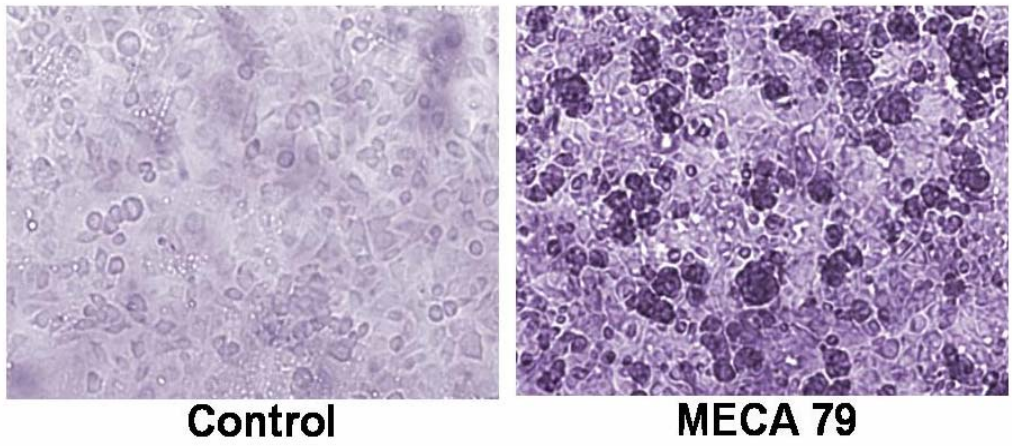


Figure 21: Immunohistochemical analysis of Ishikawa Cells shows membrane surface expression of sialyl Lewis x epitope

Next, we sought to identify L-selectin ligands by probing cell lysates with antibodies against a specific ligand, MadCAM-1. MadCAM-1 antibodies which detect two different glycoforms of this protein, one at 40 kDa and one at 60 kDa were used. In figure 22, representative Western Blot results for *one test set* are shown, for each antibody, as well as the actin (total protein) loading control.

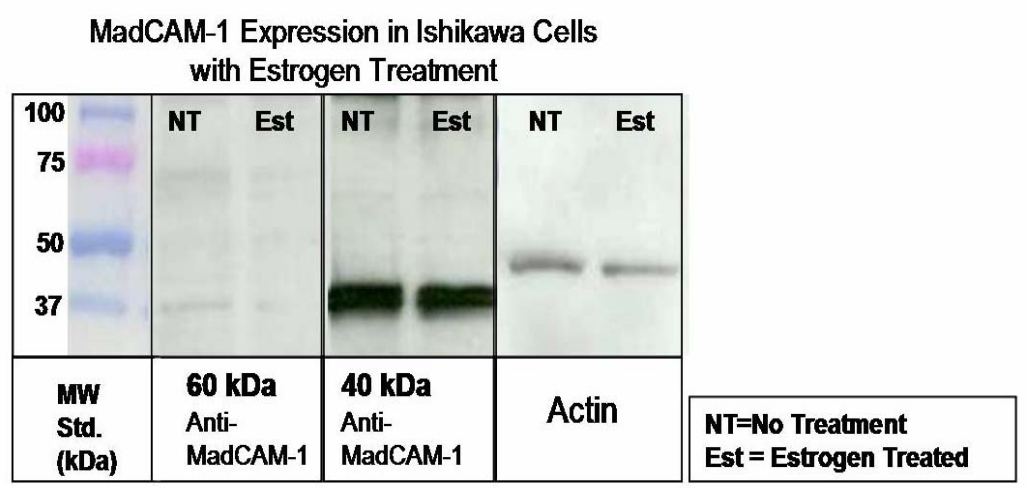


Figure 22: Representative western blot for 40 kDa and 60 kDa MadCAM-1 detection in Estrogen Treated Ishikawa cells

In Figure 23, the relative expression of 40 kDa MadCAM-1 with hormonal treatment in Ishikawa cells is shown.

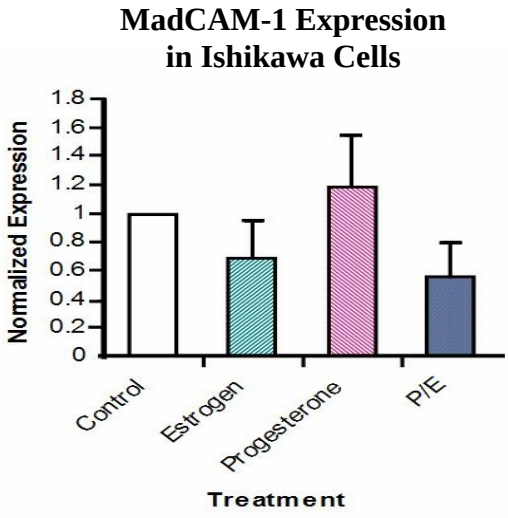


Figure 23: Normalized expression of 40 kDa MadCAM-1 in Ishikawa Cells

3.4 Discussion

It has been established that the primary factors that stipulate endometrial receptivity in humans are the ovarian steroids, estrogen (E2), and progesterone (P4). Biomedical research in animal models has provided great insight into the process of human reproduction, however, in the case of implantation, the human process differs significantly and no suitable animal model has been found. The work reported herein has been performed with the intention of identifying a suitable cell model capable of being utilized for further detailed studies of the implantation process, with a specific focus on the function of the L-selectin, thought to be the *initial* cell adhesion protein involved in the attachment of the floating blastocyst to the uterine epithelium. We therefore identified possible candidates from existing trophoblast cell lines. The selection criteria required that these cells were derived from first trimester trophoblasts, since the protein expression profile of trophoblast cells *in vivo* is known

to change significantly as the pregnancy progresses, to the extent that by 17 weeks gestation, trophoblast staining for L-selectin is virtually absent [141]. The candidate cell lines were JAR, Jeg-3 and BeWo cells. Based on the work of Lessey [81] we established a protocol for preparation of cell lysates to be used in examining basal expression of L-selectin in these cells through western blot analysis, as well as its modulation in a hormonal environment which simulated the temporal expression levels of the primary ovarian steroid hormones, estrogen and progesterone. The presence of estrogen mimics the follicular phase of the menstrual cycle. It was hypothesized that the cellular expression of L-selectin in this environment would be reduced as compared to an environment of progesterone, which is the hormone that dominates in the luteal phase. The window of implantation is a 2-3 day period of time when it is suspected that all conditions which constitute uterine receptivity towards an implantation competent blastocyst are present, and this includes known levels of both estrogen and progesterone. Thus, it was hypothesized that the expression of L-selectin would ideally be highest in a hormonal environment that mimicked the window of implantation. Qualitatively, immunoblot analysis using the L-selectin-specific antibody Dreg-56 verified L-selectin expression in all three cell lines (15 and 16A). The monocytic leukemia cell line U-937, which has been described to express L-selectin, was used as positive control [156]. Upon hormonal stimulation with progesterone, estrogen or a combination of both, JAR cells upregulated L-selectin under all conditions (figure 16A), however, this response was found to be significant ($p<0.05$) only in the environment of both hormones, which mimics the window of

implantation. BeWo cell L-selectin expression was only affected when stimulated with estrogen (downregulation of about 40%). Jeg-3 cells responded with 30% downregulation of L-selectin to estrogen and upregulation of about 30% after combination treatment with progesterone and estrogen; however, these differences were not statistically significant ($p>0.53$).

In addition to global L-selectin expression, we also determined cell surface expression of the molecule on each of these cell lines by flow cytometry, again using the U937 cell line as a positive control. We were unable to detect L-selectin on the surface of BeWo or JAR cells. Therefore, we concluded that it is unlikely that L-selectin plays a role in initial uterine attachment of these cells and excluded them as a blastocyst model. *However, Jeg-3 cells were clearly positive for cell surface expression and therefore were selected for further studies.* Thus, although Jeg-3 cells are cancer cells, they have preserved responsiveness to endocrine regulators. Nevertheless, caution should be used in trying to extend these results to trophoblast cells.

Since Ishikawa cells have been particularly well characterized with regard to the expression of protein biomarkers of uterine receptivity, they were used exclusively as the cell model for uterine epithelial tissue. An important characteristic of any candidate cell line in this study was that they maintain estrogen and progesterone receptors. Their presence has been demonstrated in the Ishikawa cells [325] and the results presented in this work for the candidate trophoblast cell lines would also support the presence of estrogen and progesterone receptors.

Though it is hypothesized that the Ishikawa cells, as an appropriate model for the uterine epithelial tissue, will express L-selectin ligands, the question remains as to whether or not these cells may also express L-selectin. We felt that it was reasonable to explore this question for two reasons. First, a cell can secrete signal molecules that can bind back to its own receptors in a process called autocrine signaling [119]. Autocrine signaling has been reported in the implantation process with regard to the expression of Fas/Fas Ligand (a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family) in the human endometrium [329]. Second, as is the case with the trophoblast cell lines, Ishikawa cells are carcinoma cells and as such they often use autocrine signaling to overcome the normal controls on cell proliferation and survival [119]. Thus, Ishikawa cells were evaluated for both basal L-selectin expression and its modulation in the presence of the ovarian steroid hormones. We found basal expression of L-selectin, as well as its modulation by a combination treatment with progesterone and estrogen (slight upregulation of about 30%). This novel finding is interesting, and does support the hypothesis of an autocrine signaling process which may result in the expression of both L-selectin and its ligands on the same cell type. Whether this interesting and novel finding is representative of events which occur in the actual process of implantation, or is an artifact of the fact that this is an adenocarcinoma cell line remains to be verified by further investigation.

The MECA 79 antibody recognizes sulfate and carbohydrate epitopes on an important class of endothelial ligands. It was a key component in the finding of L-selectin

ligand upregulation on uterine epithelial tissue during the window of implantation [141]. In this work, MECA-79 antibody recognized several proteins on western blots with molecular weights ranging from less than 10 to 100 kDa (Figure 19), indicating that Ishikawa cells express several proteins with the sialyl-Lewis *x* modification. We also investigated the response of Ishikawa cells to hormonal stimulation with respect to expression of sialyl-Lewis-X proteins by quantitative western blots. The total intensity of the bands, over the range of 10-100 kDa, as well as four of the most prominent bands at MW 40, 50, 80 and 90 kDa, were quantified by the image analysis technique earlier described (Section 3.3); the 90, 50 and 40 kDa were selected because they corresponded to the molecular weight of known L-selectin ligands (CD34, GlyCAM-1 and MadCAM-1), and the 80 kDa band was included because it was one of the most prominent bands (Figure 19). Little response was observed for the 40 and 50 kDa proteins, regardless of the treatment. The 80 and 90 kDa proteins were significantly suppressed, with the combination treatment achieving the strongest effect. Thus, basal expression of non-specific MECA-79 reactive L-selectin ligands in Ishikawa cells has been confirmed. Given the fact that the presence of estrogen and progesterone receptors in this cell line has been confirmed by other researchers [325], it would be reasonable to speculate that modulation of the ligand expression by ovarian steroids would be present, however, it was not statistically significant. This may be due to the limited number of experiments which were performed (n=3 for all conditions), or it may be a result of many passages in the maintenance of this cell line. Transformed cells such as the Ishikawa cells, that

become established in culture can undergo karyotypic changes, usually marked by an increase in chromosomes (polyploidy), with continual passage [330]. Furthermore, L-, P- and E-selectin ligand expression in carcinoma cells (typically those which are ready to metastasize) is sometimes seen, as this expression is thought to be a part of the mechanism of extravasation of the metastasizing cell, similar to the process of leukocyte extravasion in response to normal inflammatory processes [331-333]. The latter two conditions are unlikely, since Ishikawa cells have been well established as a stable immortalized cell line, with protein expression characteristics, particularly integrins, which mimic normal uterine tissue [325]. *The constitutive expression of MECA 79 reactive L-selectin ligands, which was also verified by immunohistochemistry (Figure21), is a novel and important finding which will allow this cell line to be utilized as a cell model in future experiments.*

We sought to further identify specific L-selectin ligands by probing cell lysates with antibodies against the known ligands, in particular, MadCAM-1. Since trophoblast attachment and implantation into uterine epithelium has been proposed to mimic leukocyte attachment and extravasation through the vascular endothelium [142] we hypothesized that Ishikawa cells would only express the 40 kDa glycoform of MadCAM-1 in analogy to vascular endothelium. Confirming this hypothesis, a strong signal was detected using an antibody to the 40 kDa glycoform of MadCAM-1 by immunoblot analysis, while an antibody against the 60kDa glycoform did not react (Figure 22). Estrogen treatment induced a downregulation of 40 kDa MadCAM-1 expression of about 30% (Figure 23). Overall, these experiments support the

hypothesis that L-selectin ligand expression in Ishikawa cells is modulated by hormones.

In summary, this work has resulted in the establishment and validation of an appropriate cell model for further studies of the process of initial human implantation. Jeg-3 and Ishikawa cell lines have shown protein expression characteristics which are consistent with those of human tissue, that is, L-selectin expression on the trophectoderm of an implantation ready blastocyst, and L-selectin ligand expression in uterine epithelial tissue, respectively. Furthermore, the novel findings of L-selectin expression in the Ishikawa cells, and the specific L-selectin ligand, MadCAM-1, has raised interesting new questions about protein adhesion mechanism in implantation which can be explored in future work.

CHAPTER 4: L-SELECTIN MODULATION UNDER EXOGENOUS INFLUENCES OF GLUCOCORTICOIDS AND HYDRODYNAMIC SHEAR FLOW

4.1 Introduction

In the quest to improve outcomes in Assisted Reproductive Technology (ART), clinical interventions have focused on either the "embryo factor", or the intra-uterine environment. There have been few studies aimed at improving the "embryo factor" in implantation. These include the establishment of guidelines for "high quality embryo selection" [334], pre-implantation genetic screening [335], optimizing techniques for embryo transfer [336], and assisted hatching (artificial disruption of the embryo's outer coating), the latter of which has been most widely advocated [337, 338]. However, in 2005, a review of the efficacy of this treatment concluded that there is little evidence to support its benefits in overcoming implantation failure [339]. Supplementation of the *in-vitro* fertilized embryo culture medium with physiologically occurring cytokines [15, 16] and exogenous steroids [14] has also been reported to improve the implantation success rate; however, no comprehensive review of this treatment genre has been done to determine its overall effectiveness. In recent years, there has been much attention focused on the intra-uterine environment for therapies which will improve the rate of successful implantation and pregnancy [336]. Uterine receptivity is controlled by locally acting growth factors and cytokines [24] as well as natural killer (NK) cells, which have been shown to play an important role in early implantation [340]. Flaws in the integrity of the cytokine system, as well as an excess of NK cells, have been implicated in implantation failure [341,

342]. It has therefore been proposed that glucocorticoids (hormones with potent anti-inflammatory and immunosuppressive properties) may serve to improve the "receptive" uterine environment by acting as immuno-modulators to reduce the NK cell count, normalize the cytokine expression profile, and reduce endometrial inflammation [336]. Additionally, women with the condition of Poly Cystic Ovarian Syndrome (PCOS) are known to have a higher than normal level of androgens, and this is suspected to be detrimental to both oocyte quality and implantation success in IVF treatments. Glucocorticoid administration has been utilized in women with this condition to reduce androgen concentrations, but with limited success [343].

Several different protocols for glucocorticoid administration have been used, where dosing and/or length of therapy have been varied. Treatments have been given in the luteal or follicular phase or both [344-346] and two IVF studies have reported administration of glucocorticoids from embryo transfer onwards [347, 348]. The glucocorticoids used for treatment of subfertile women have included: (methyl) prednisolone [345, 347-353], hydrocortisone and (methyl) prednisolone in combination [346] dexamethasone [344] and hydrocortisone and dexamethasone in combination [354].

In a recent review, the results of several studies, focused on peri-implantation glucocorticoid administration *as a routine treatment* in assisted reproductive technology (ART), were carefully analyzed [336]. The authors concluded that there was no clear evidence that routine administration of glucocorticoids in ART cycles improves the clinical outcome [336]. However, they cautioned that these conclusions

could not be extrapolated to women with unexplained infertility, endometriosis or those with high levels of uterine NK cells. Thus, they recommended that, in order to clarify the role of glucocorticoids in aiding implantation, further trials focusing on women with these conditions (i.e. well defined subgroups) should be performed.

The actions of glucocorticoids in the human organism are well characterized. Synthetic glucocorticoids possess the ability to bind with the cortisol receptor and trigger effects similar to their naturally occuring counterparts (eg, cortisol). Glucocorticoids are distinguished from mineralocorticoids and sex steroids by the specific receptors, target cells, and effects. Cortisol (or hydrocortisone) is the *most important* human glucocorticoid. It is essential for life, and it regulates or supports a variety of important cardiovascular, metabolic, immunologic, and homeostatic functions. Glucocorticoids influence all types of inflammatory events, no matter what their cause.

The most widely used synthetic glucocorticoids are prednisone, prednisolone, methyl prednisolone and dexamethasone. Cortisol (hydrocortisone) is the standard of comparison for glucocorticoid potency, thus the potency of synthetic hormone is measured as a factor of its effectiveness with relation to cortisol. Table 1 shows the relative potency of these important glucocorticoids [355].

Table 1: Relative potencies of important synthetic glucocorticoid compounds

Name	**Potency**	**Duration of action ($t_{1/2}$ in hours)**
Cortisol (hydrocortisol)	1	8
Prednisone	3.5 - 5	16- 36
Prednisolone	4	16- 36
Methyl prednisolone	5 – 7.5	18 - 40
Dexamethasone	25 – 80	36 – 54

The action of glucocorticoids is a function of their structure which facilitates their passage across the cell membrane, where they are recognized by cytosolic glucocorticoid receptors. Glucocorticoid receptors are found in the cells of almost all vertebrate tissues. The structure of dexamethasone is based on the cortisol structure but differs in three positions (extra double bond in the A-ring between carbons 1 and 2 and addition of a 9-α-fluoro group and a 16-α-methyl substituent). Figure 23 shows the structure of cortisol, and two glucocorticoids of importantance in reproductive therapies, dexamethasone and prednisolone.

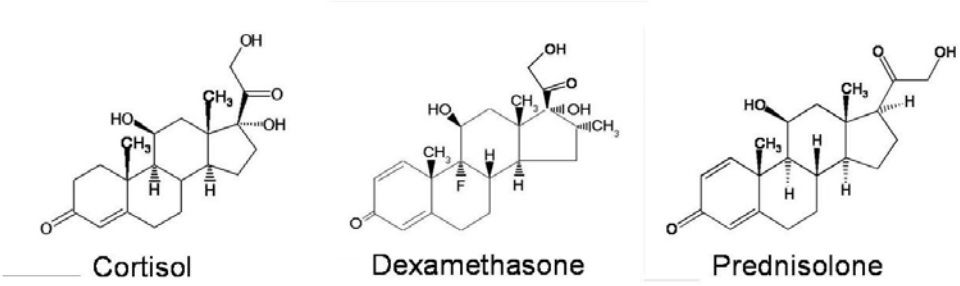


Figure 24: Chemical Structures of Cortisol, Dexamethasone and Prednisolone [355]

The inhibition of leukocyte accumulation in inflamed tissues is one result of the anti-inflammatory effect of glucocorticoids. One mechanism that has been suggested is that glucocorticoids, at clinically relevant concentrations, exert specific actions on *expression* of adhesion molecules on activated neutrophils, which are mediated through *ligation* of glucocorticoid receptors [356]. Another mechanism is that glucocorticoids, specifically dexamethasone, can act to up regulate and externalize annexin, a calcium binding protein typically found in granulocytes and monocytes, which can then co-localize with L-selectin, and facilitate *its shedding* through calcium dependant interaction, thereby interfering with leukocyte tethering to vascular endothelium and subsequent extravasation [156]. Given the many similarities between leukocyte attachment/extravasion and blastocyst attachment/implantation, in particular the initiation of these events with L-selectin/L-selectin ligand interactions, *it would be useful to investigate the effect of glucocorticoids on L-selectin in a trophoblast cell model.* The observations could provide insight into the underlying reasons for the apparent failure of glucocorticoid administration in ART cycles to positively affect the clinical outcome.

Neutrophils have been shown to increase L-selectin expression in the presence of fluid flow [162] Further, in the presence of fluid flow, L-selectin in the neutrophil membrane has been demonstrated to redistribute and localize, forming a “cap” or polarity of expression in the cells [171]. This group suggests that the capping of L-selectin and it’s ligand, PSGL-1, is accomplished by an active transport process, a hypothesis which is supported by the fact that the speed of redistribution of receptors

to form a cap would require a 10-100 fold greater speed than previously reported for passive diffusion of integral membrane proteins [357]. The membrane capping of L-selectin in neutrophils is thought to provide an important role in the mechanics of adhesion while the cells are in a flow environment. *This same effect is not seen under static conditions,* where L-selectin remains homogeneously distributed throughout the neutrophil cell membrane [171].

Similarly, while the specific fluid mechanics of the uterine cavity have not been fully described, it can be assumed that there is at least a mixing flow provided by asynchronous contractions [176] and mucin secretions by the endometrium [141]. There may also be a more oriented flow resulting from beating of cilia on the endometrial ciliated cells. Cilia-mediated flow has been shown to provide directional flow in the oviduct [173, 174]. The effect of fluid flow on trophoblast L-selectin expression has not been previously described. However, because of the possible clinical treatments that could be implemented with the use of flow to stimulate L-selectin expression, it would be of great interest to explore the modulation of L-selectin in trophoblast-like cells, under conditions of flow.

The effect of dexamethasone or fluid flow on L-selectin *ligand* expression in the uterine epithelium has not been studied. In fact, few studies exist which focus on L-selectin ligand modulation with dexamethasone. It has been reported that dexamethasone can inhibit the TNF-α induced up-regulation of MadCAM-1 [242], but it has no effect on the regulation or expression of PSGL-1 on neutrophils [226].

Thus, in addition to modulation of the L-selectin adhesion system by the local environment of sex steroid hormones and cytokines, we hypothesize that this system is further modulated by inflammatory mediators *as well as* mechanical shear forces due to flow. The work reported in Chapter 3 has resulted in the characterization and validation of an optimized cell model consisting of trophoblast-like Jeg-3 cells and uterine epithelial-like Ishikawa cells. This cell model has been shown to be responsive to the primary sex steroid hormones, estrogen and progesterone. The work reported in Chapter 4 will focus on the quantification, through western blot analysis, of expression and modulation of L-selectin and L-selectin ligands in the presence of the powerful anti-inflammatory agent, dexamethasone, and as a response to hydrodynamic shear forces. Furthermore, L-selectin shedding in response to these two conditions will be quantified with the use of ELISA.

4.2 Materials and Methods

Hormonal Conditioning of Cells

Jeg-3 and Ishikawa cells were selected as the optimum cell model, as a result of the work reported in Chapter 3. The culture conditions, and passaging protocol are reported in section 3.2., *Cell Lines and Cell Culture*. Hormonal treatment of the cells was initiated after the cells were passaged and incubated for 24h under normal conditions, to assure log-growth properties. Cells were then placed in phenol red free culture medium: D-MEM F-12, *#21041* (*Gibco/Invitrogen)* supplemented with 10% charcoal stripped FBS to eliminate background stimulation by estrogen-mimetics or FBS-derived hormones. Additionally, cells were treated with physiological

concentrations of dexamethasone, 10^{-7} M (*Thermo Fisher Scientific, Pittsburgh, PA*) for 24 or 48h.

Exposure of Cells to Hydrodynamic Shear Forces

Jeg-3 cells were first passaged and incubated for 24h with normal culture medium, and under static conditions in order to assure log-growth properties. Cells were then washed and the culture medium replaced with phenol red free culture medium: D-MEM F-12 supplemented with 10% charcoal stripped FBS. At this point, the test samples were subjected to random fluid flow by placing the culture flasks on a standard laboratory rocker, cycling at 3.25 cycles/min. Test cultures on the rocker, along with static control cultures, were incubated at 37^{o}C for 24 hours. Figure 24 shows the experimental setup. Cells and supernatant were collected for analysis of cellular as well as shed L-selectin.

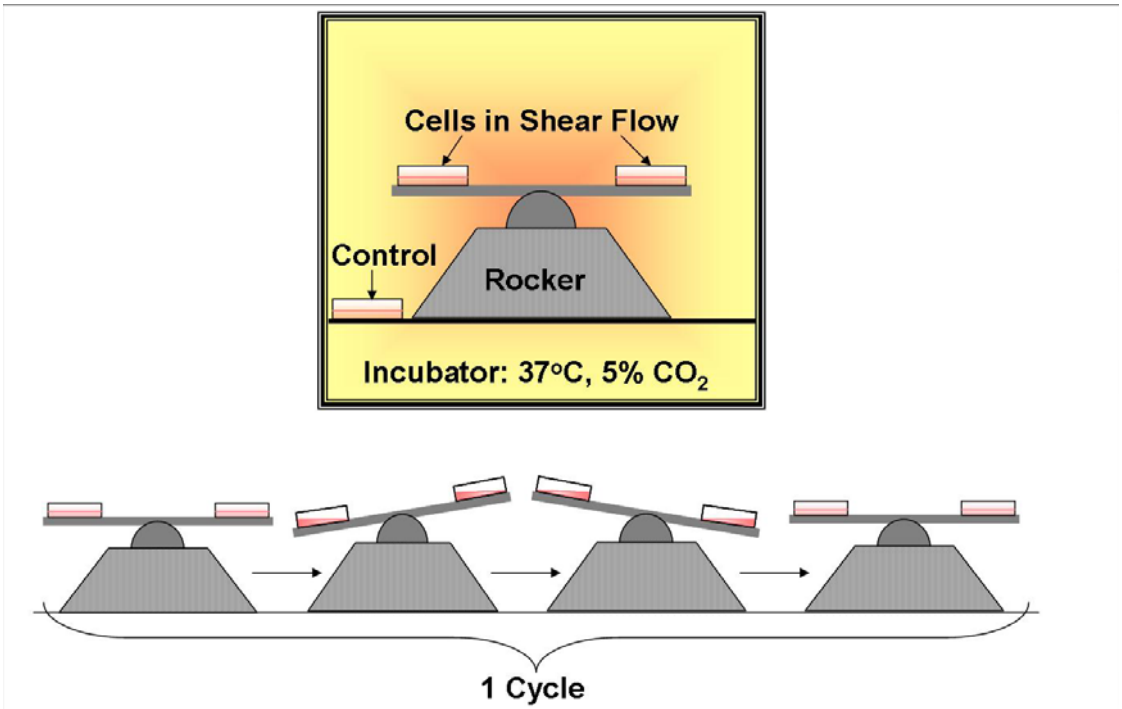


Figure 25: Experimental setup for conditioning Jeg-3 cells in random hydrodynamic shear flow.

Antibodies and Antigens

For western blot analysis of L-selectin modulation, as well as the preparation of standards for ELISA, both recombitant human L-selectin FC chimera #728-LS (*R & D Systems (San Diego, CA*), and U 937 cells were used as positive controls. The primary antibodies used for western blot experiments were: LEAF purified anti-human CD62L (L-selectin) (DREG-56) from *BioLegend* (*San Diego, CA*), MECA-79 rat anti-mouse monoclonal antibody from *BD Pharmingen* (*San Jose, CA)*, mouse anti-human mucosal addressin cell adhesion molecule-1 (MAdCAM-1) obtained from *Serotec, Raleigh, NC (# MCA2096Z),* and β-Actin rabbit polyclonal antibody from

Cell Signaling Technology, Inc. *(Danvers, MA*). The quantification of actin in the cell lysates provided the internal loading control.

The ELISA antibodies and reagents were: N-18 L-selectin polyclonal antibody (*Santa Cruz Biotechnology, Santa Cruz, CA*), DREG-56 (*BioLegend),* biotinylated goat-anti mouse secondary antibody #BA-9200 (*Vector Labs, Burlingame, CA*), HRP conjugated Streptavidin, #21126 and TMB (tetramethyl benzidine) Substrate Kit, #1854050, (both from *Pierce, Rockford, IL*).

Supernatant and Cell Lysate Preparation

For quantification of cellular L-selectin modulation through western blot, cells were harvested and processed into lysates using the same protocols described in section 3.2, "*Cell Lysate Preparation and Western Blotting"*.

The supernatant was processed for quantification of *shed* L-selectin as follows. Cellular debris was removed by centrifugation at 1000 rpm for 10 minutes, followed by syringe filtration through a 0.2 uM filter. Media were concentrated in a two step process: Centriplus centrifugal protein concentrators #YM-30 with a molecular weight cutoff of 30, 000, were first used for 15-20 fold concentration, followed by Microcon #YM-30 for concentration to a final range of 40-65 X (both from *Millipore Corp., Bedford MA)*. All supernatants were kept at 4^{o}C or lower (when possible) during this process. Concentrated supernatants were stored at -80^{o}C. .

ELISA for Shed L-selectin

Plates were coated overnight at 4^{o}C with 3 ug/ml N-18 polyclonal L-selectin antibody. All subsequent steps were done at room temperature. Coated wells were

blocked for 2h with 3% BSA in PBS. Samples and standards, prepared in phenol red free culture medium with 10% charcoal stripped FBS, were incubated for 2h. The captured L-selectin was detected with DREG-56 monoclonal antibody (1:500), incubated for 1 hour followed by biotinylated goat anti-mouse antibody (4ug/ml) incubated for 1 hour. Staining was performed with HRP conjugated streptavidin (0.5 ug/ml) and TMB substrate system, and stopped in 3 M HCl. For quantification, absorbance measurements were made at 450 nm on a microplate reader. A detailed protocol for supernatant (culture medium) concentration and ELISA can be found in Appendix F.

4.3 Results

Western Blot Detection of Cellular L-selectin

Figure 26 shows representative western blots for treated Jeg-3 (A) and Ishikawa (B) cells at 24 and 48 hours of dexamethasone exposure. The blots were quantified for L-selectin expression and modulation, using corresponding actin bands as total protein loading controls. Cellular L-selectin is expressed at ~70 kDa. Actin is expressed at 40 kDa. Positive and negative controls were used for all western blots, as previously described (Chapter 3). The blots show single sample sets, with each set consisting of 1 lysate of treated cells, and 1 lysate of non-treated cells. In all cases n=3, unless noted otherwise.

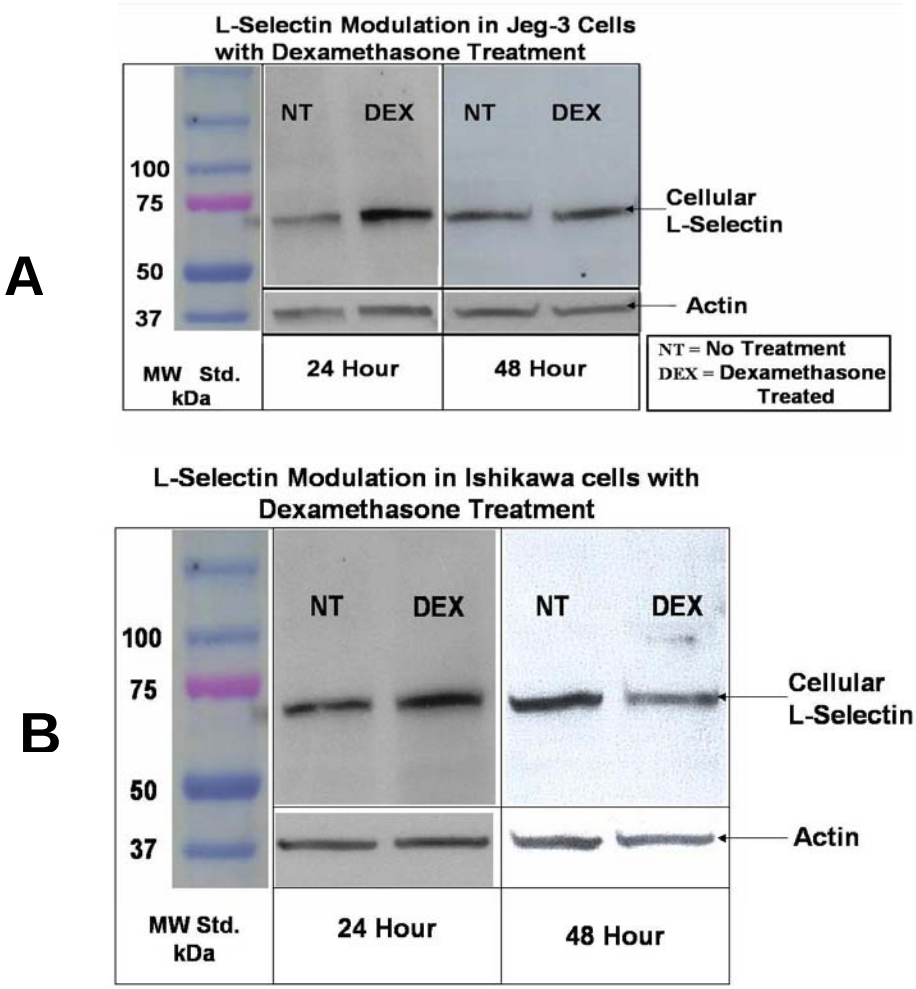


Figure 26: Representative Western Blots showing L-selectin expression and modulation in the presence of dexamethasone in Jeg-3 (A) and Ishikawa (B) cells

The procedure and calculations used to quantify relative expression of L-selectin have been presented in Section 3.3. Cellular L-selectin expression in Jeg-3 and Ishikawa cells, as well as the temporal modulation of L-selectin expression with dexamethasone is shown in Figure 27. For the 48 hour dexamethasone treatment of Jeg-3 cells, n = 6.

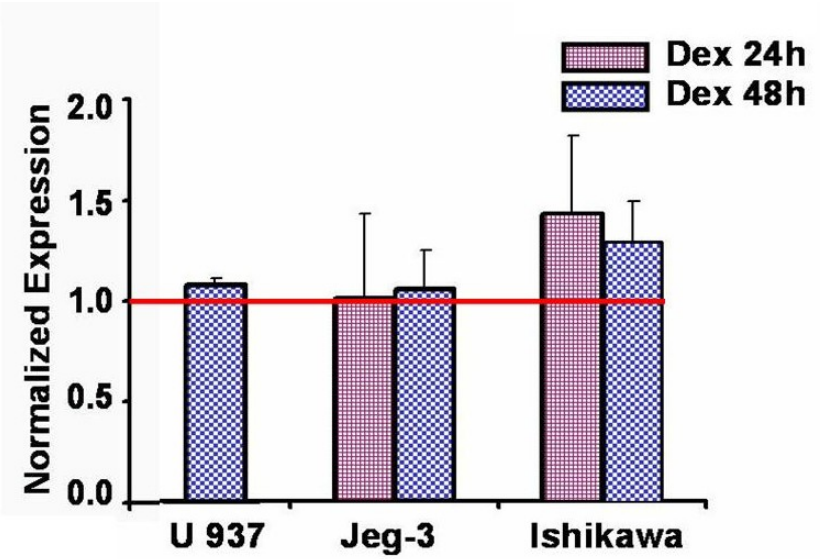
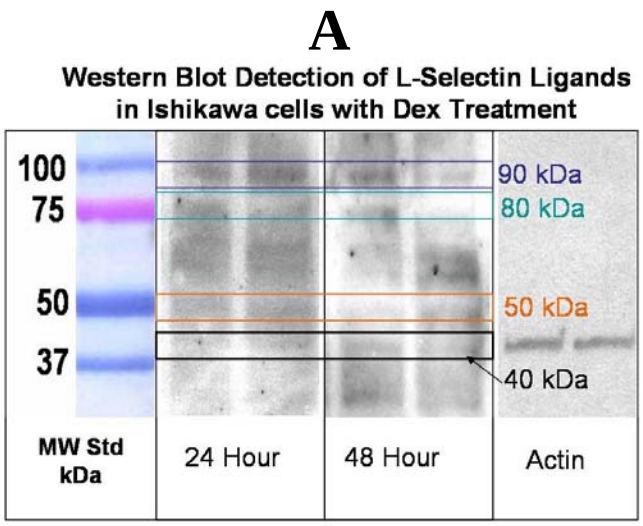


*Figure 27: Normalized **Cellular** L-selectin expression in Jeg-3 and Ishikawa cells after treatment with dexamethasone for 24 and 48 hours. U 937 cells were used as a positive control, at 48 hrs only. Quantification by western blot.*

Therefore, the temporal expression of sialyl Lewis X oligosaccharide containing L-selectin ligands (A), and MadCAM-1 (B) with 24 and 48 hour exposure to dexamethasone, was investigated. In figure 28 representative western blots are shown.



B

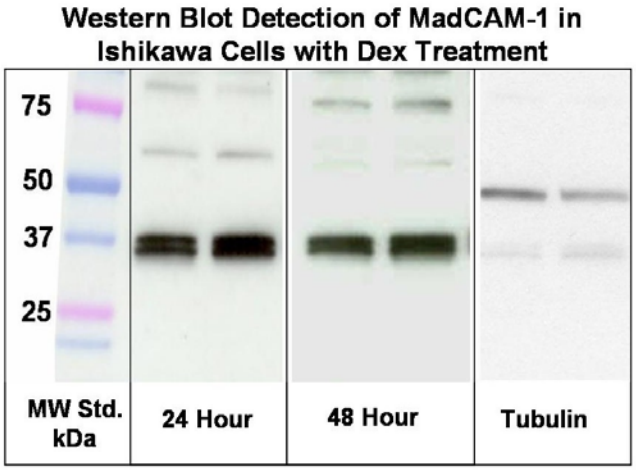


Figure 28: Western Blots showing sialyl Lewis x oligosaccharide containing L-selectin ligands (A) and MadCAM-1 (B) expression in Ishikawa cells. For clarity, tubulin (50 kDa) was used as the loading control in place of actin (40kDa).

Expression of the sialyl Lewis *x* oligosaccharide containing L-selectin ligands was quantified by the same procedure presented previously (*3.3 Detection of L-selectin*). With MECA-79 antibody, the intensity of the protein bands at 90, 80, 50 and 40 kDa, along with the "total intensity" readings were used to determine the relative expression of L-selectin ligands. "Total intensity" is defined as the intensity of all the bands, over the range of 10-100 kDa. The relative expression of the proteins at these molecular weights, as well as that for 40 kDa MadCAM-1 are presented below (Figure 29)

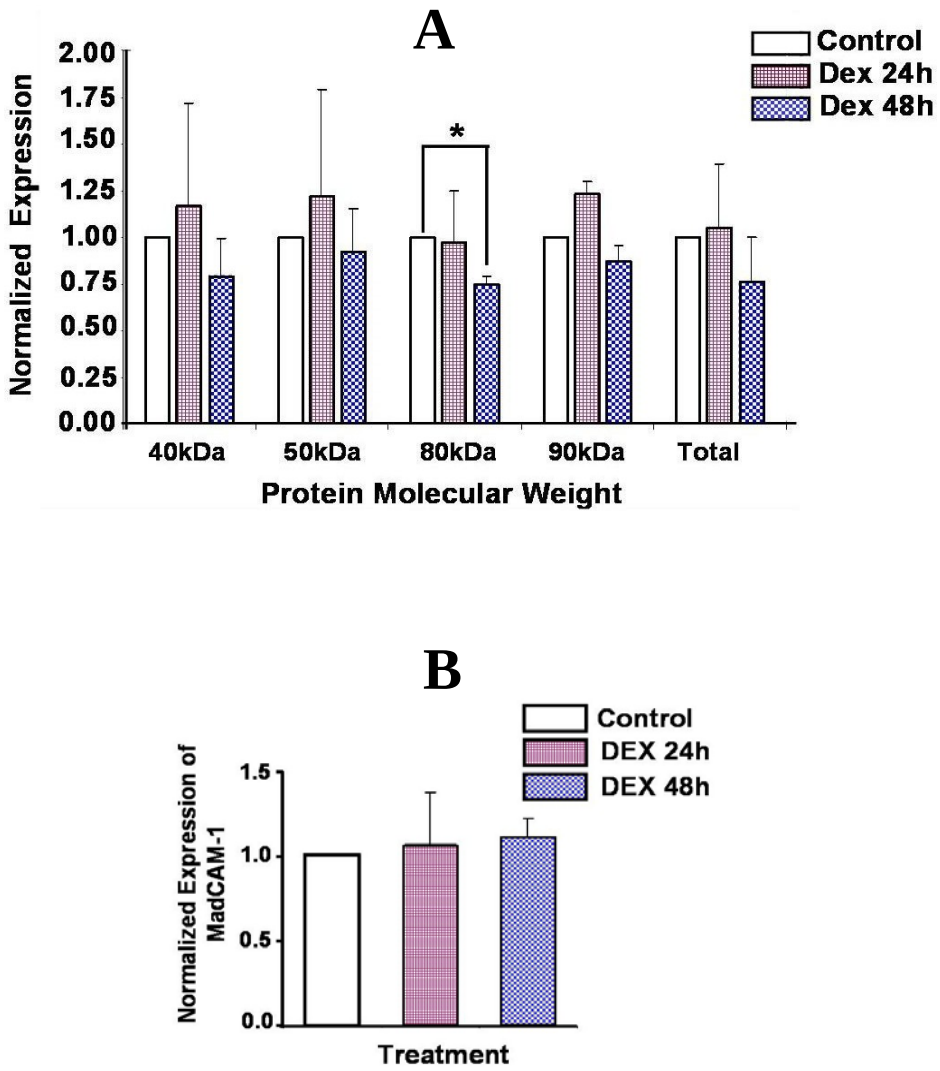


Figure 29: Temporal modulation of sialyl Lewis x oligosaccharide containing L-selectin ligands, with dexamethasone treatment, as detected by MECA 79 antibody (A) and anti-MadCAM-1 (B)

The results of ELISA analysis of the supernatant, to quantify the amount of shed L-selectin with dexamethasone treatment, are shown in Figure 30.

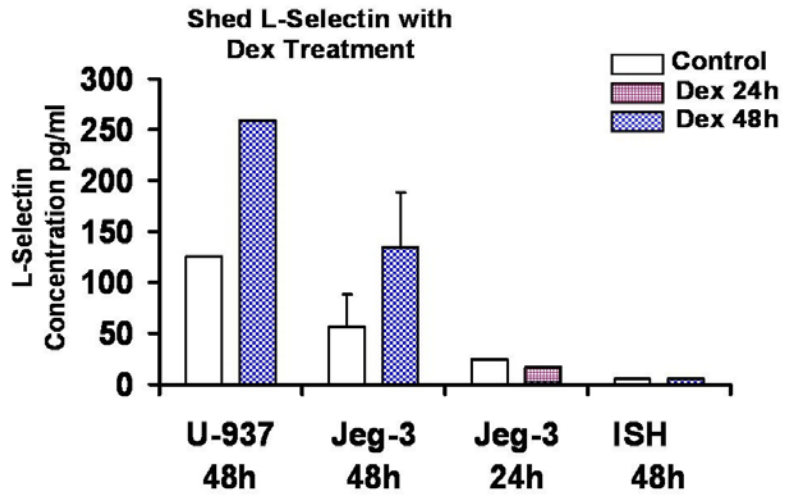


Figure 30: Quantification of shed L-selectin in the supernatant of U 937(positive control), Jeg-3 and Ishikawa cells through ELISA Analysis

In figure 30 the modulation of L-selectin in the presence of fluid flow are shown. We quantified both the levels of cellular expression through western blot, and shed L-selectin through ELISA.

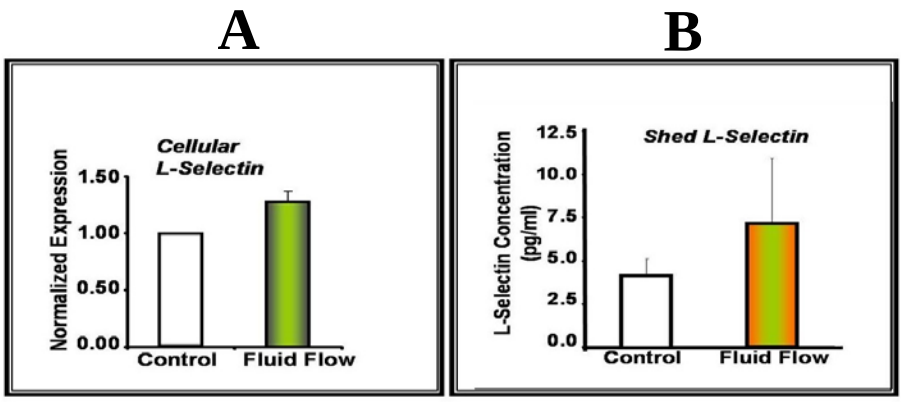


Figure 30: Effect of Fluid flow on cellular L-selectin expression in Jeg-3 cells (A), as well as L-selectin shedding (B)

4.4 Discussion

Effect of Dexamethasone on L-selectin Expression

Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid hormones which acts as an anti-inflammatory mediator and immunosuppressant. Dexamethasone and other glucocorticoids such as prednisone, prednisolone and methyl prednisolone have been used to treat infertility as a routine step in IVF treatment protocols, and in the context of inflammatory disease, such as PCOS [358, 359] but with unproven success. The glucocorticoid effect on expression of components of the L-selectin adhesion system in the reproductive process is not well-understood. With L-selectin, two mechanisms of down regulation have been proposed. The first is that glucocorticoids, at exert specific actions on expression of adhesion molecules on activated neutrophils, which are mediated through *ligation* of glucocorticoid receptors [356]. The second is that glucocorticoids, specifically dexamethasone, can act to upregulate and externalize annexin, a calcium binding protein in granulocytes and monocytes, which can then co-localize with L-selectin, and facilitate its shedding through calcium dependant interaction, thereby interfering with leukocyte tethering to vascular endothelium and extravasion [156]. The latter mechanism seems to be the most widely accepted.

Since dexamethasone is the most potent of the commonly used glucocorticoids (Table 1), and it was used to elicit both of the proposed mechanisms of down regulation, we analyzed the effect of physiological levels of dexamethasone on Jeg-3 and Ishikawa cells by quantitative western blot, and quantified the levels of

sL-selectin in the corresponding cell supernatant. U 937 cells were used as a positive control. From figure 23, it appears as if the relative expression of **cellular** L-selectin in U 937and Jeg-3 cells is only slightly affected, even after 48 hours, but Ishikawa cells show a much higher level of upregulation; almost 50% after 24 hours. If the proposed mechanism of dexamethasone induced shedding [156] is accurate, then the ELISA results for the dexamethasone treated cells should reflect this by showing a correspondingly higher level of shed L-selectin in the supernatant than the untreated cells. *Indeed this is true for the U 937 cells (which were evaluated only at 48 hours) as well as the Jeg-3 cells at both 24 and 48 hours* which raises the question of whether dexamethasone, by inducing ectodomain shedding, has any effect, positive or negative, on initial blastocyst attachment. Increased shedding may also lead to altered rolling velocity of the blastocyst, which is controlled by shedding activity in leukocytes. However, the effect of dexamethasone on L-selectin expression and shedding should be further investigated in the presence of fluid flow and other hormonal conditioning.

The Ishikawa cells not only show virtually no difference in the level of shed L-selectin but on an **absolute basis**, very little shed L-selectin is seen at all. This can be seen by comparison, in Table 2.

Table 2: Amount of s L-selectin in cell supernatant as determined by ELISA

	Shed L-selectin after 48 hrs. (pg/ml)	
Cell line	**Control (No Treatment)**	**Dexamethasone (10^{-7} M)**
U 937	125	259
Jeg-3	56	134
Ishikawa	5.02	5.11

For both U 937 and Jeg-3 cells, the relative increase in sL-selectin with dexamethasone treatment is over 100%, while in the case of the Ishikawa cells, the sL-selectin level is one order of magnitude lower than the lowest level seen in the Jeg-3 cell line, and more than 2 orders of magnitude lower than that of the U 937 positive control. In considering both the upregulation of L-selectin in the Ishikawa cells, as determined by western blot, and the apparent lack of shedding, it is consistent to conclude that the cellular L-selectin in Ishikawa cells may be primarily found within the cell, and not as a trans-membrane type protein, as with the Jeg-3 cells. However it must also be considered, that the L-selectin may be expressed on the cell membrane, but in such a configuration that it was not accessible to the antibodies used in flow cytometry, nor to the proteases that promote L-selectin shedding. Furthermore, Ishikawa cells do express L-selectin ligands (Chapter 3). It has been suggested by Khan, et al [177] that one anti-inflammatory mechanism in lymphocytes is the binding of sL-selectin to presenting ligands preventing further interaction with membrane bound L-selectin. This would also be consistent with an apparent

upregulation of cellular L-selectin. Further testing would need to be done in order to fully characterize the function of L-selectin in Ishikawa cells.

Effect of Dexamethasone on L-selectin Ligands

Though many studies have been reported regarding the effect of dexamethasone on L-selectin, little is known of its effect on L-selectin ligand expression and modulation. It has been shown that L-selectin is shed from the membrane surface as a result of dexamethasone exposure[156] , and that soluble (shed) L-selectin can retain its functional activity [157]. It has been suggested that soluble L-selectin (sL-selectin) can regulate lymphocyte migration into the peripheral lymph nodes with increased levels (of sL-selectin) resulting in more profound inhibition of this process [177]. The presumed mechanism is one in which sL-selectin binds to the presenting ligands and prevents further interaction with membrane bound L-selectin on lymphocytes. With the finding of L-selectin expression in Ishikawa cells, it is reasonable to hypothesize that, under the influence of dexamethasone, a mechanism might exist in which shed L-selectin might effect L-selectin ligand expression in the same cells.

The effect of glucocorticoid treatment on temporal expression of L-selectin ligands in Ishikawa cells was therefore examined by quantitative immunoblot in a similar manner to the protocol given in Chapter 3. In quantifying the MECA 79 reactive ligands, the bands at 90, 50 and 40 kDa were selected because they correspond to the molecular weight of known L-selectin ligands (CD34, GlyCAM-1 and MadCAM-1), and the 80 kDa band was included because it was one of the most prominent bands,

though no specific L-selectin ligand has been reported to correspond to this MW. After 24 hours of exposure, cells responded with a moderate but insignificant upregulation of L-selectin ligands, with the exception of the 80 kDa protein. Cells responded to prolonged (48 hr) dexamethasone exposure with a **reduction** of expression of all four proteins examined, in particular, the reduction in the 80kDa band (about 25%), was statistically significant ($p=0.03$) (Figure 29A). Very little is known of the modulation of L-selectin ligand expression with dexamethasone. Only one study has been reported, where the regulation of PSGL-1 on monocytes by dexamethasone was examined, and it was unaffected by the dexamethasone treatment [226]. In contrast, Genbacev et al., have shown that the attachment of the blastocyst to the uterine wall is mediated, at least in part, by (MECA 79 reactive) selectin oligosaccharide-based ligands. L-selectin ligands are upregulated in the endometrium during the luteal phase coinciding with the high expression of L-selectin on trophoblastic cells thus promoting the initial step of implantation. Our finding may therefore be significant in that a downregulation of the MECA 79 reactive ligands in the presence of dexamethasone could impair uterine receptivity to blastocyst attachment during the initial stages of the implantation process. This observation is also consistent with the recent review which concluded that there was no clear evidence that routine administration of glucocorticoids in ART cycles improves the clinical outcome[336]. It may in fact be detrimental to primary implantation mechanisms, however, further examination of hEEC's will be necessary to confirm this observation.

The effect of dexamethasone on **cellular** MadCAM-1 expression in Ishikawa cells was also evaluated by western blot. Figure 25 shows that dexamethasone has very little effect on the expression of this ligand. MadCAM-1 expression has not been characterized with respect to the uterine *epithelium*, where it would likely be involved in the process of implantation, but it has been observed throughout the secretory phase in the uterine endometrium[214]. Its presence was associated with selective recruitment of natural killer (NK) cells to the endometrium as a normal function in the menstrual cycle. The regulation of MadCAM-1 expression has been studied, particularly with a focus on treatments for acute and chronic inflammatory conditions of the intestine, such as Crohn's Disease and ulcerative colitis [239, 240]. MadCAM-1 has been shown to be up-regulated in murine lymph node endothelium by the inflammatory cytokine, TNF-α, [241]. Furthermore, in the human intestinal tract, TNF-α mediated upregulation of MadCAM-1 (due to inflammatory bowel disease) is *inhibited* by dexamethasone treatment [242]. Thus, it is consistent that dexamethasone would have little effect on the expression of MadCAM-1 in Ishikawa cells, particularly in an environment lacking in pro-inflammatory cytokines like TNF-α . Furthermore, since TNF-α is *not* considered to be a significant mediator in the implantation process, there would probably not be a need to investigate its influence in this process. Presently, MadCAM-1 has not been confirmed to be present on human uterine epithelial tissue, though it is one of the aims for further studies in this project. The results of this work suggests, however, that if MadCAM-1 is found, it would probably not be negatively affected by the presence of glucocorticoids.

Effect of Fluid Flow on L-selectin

It was hypothesized that when subjected to hydrodynamic shear flow, the Jeg-3 cells might respond by upregulating L-selectin, in a similar manner as has been observed on leukocytes [162, 171]. Earlier observations by flow cytometry have demonstrated that the L-selectin expressed by Jeg-3 cells is, in part, functional as a membrane bound extracellular protein. Due to experimental configuration of this test, Jeg-3 cells would not be able to "shed" L-selectin due to interactions with membrane bound ligands, as with blood leukocytes, however, we wanted to determine if the hydrodynamic shear forces alone may contribute to the shedding process. Thus, cellular L-selectin was quantified through western blotting, and the cell supernatant was quantified for sL-selectin through ELISA (Figure 31). Expression of L-selectin on the trophectoderm of an implantation competent blastocyst (after shedding of the zona pellucida) has also been demonstrated [141], however, in this environment, modulation of L-selectin due to fluid flow has not been investigated, and this study did not specify the conditions of the environment (static or dynamic) in which the L-selectin expressing trophoblast cells were maintained. Many of the experimental designs in which the effect of fluid flow is investigated correctly include interactions with L-selectin ligands [171, 225, 360-362]. However, the suggestion has been made that "hydrodynamic shear forces alone may increase expression of L-selectin" [144]. In this experiment, we attempted to *qualitatively* investigate the effect of hydrodynamic shear forces by culturing cells on a rocker plate, as shown in Figure 25. The culture medium, flowing over attached trophoblast-like Jeg-3 cells as the

rocker cycled back and forth, was meant to mimic the effect of uterine fluid on the blastocyst, although this fluid flow has not been well defined. Based on immunoblot analysis, random fluid flow induced a *30% increase in cell associated L-selectin expression* (Figure 31A). When the cell supernatant was analyzed for sL-selectin by ELISA, there was a slight increase compared to the (static) control samples, however, as with the Ishikawa cells, the absolute amount of shed L-selectin was insignificant (<0.1x) when evaluated in the context of the dexamethasone treated cells, where shedding of the L-selectin has been demonstrated. This is illustrated in figure 32.

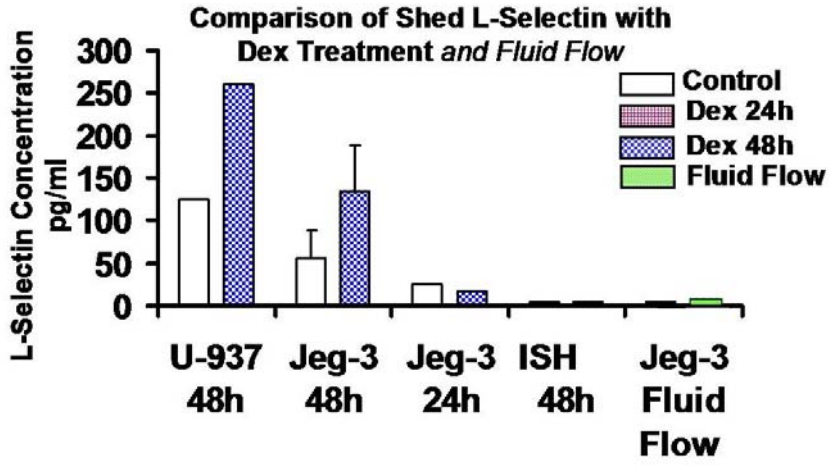


Figure 32: Comparison of shed L-selectin levels with dexamethasone treatment, and after exposure to hydrodynamic shear forces.

L-selectin shedding can occur as a result of glucocorticoid exposure [156], as a result **mechanical forces** between L-selectin and its oligosaccharide based ligands which induce the cleavage of L-selectin from the neutrophil surface during rolling and tethering to L-selectin ligands [363]. Since neither of these conditions existed in this

experiment, it is not likely that shedding of L-selectin occurred as a result of hydrodynamic shear forces that the Jeg-3 cells experienced.

This finding leads us to hypothesize that exposure of an *in vitro* fertilized blastocyst, to fluid flow of a carefully determined range of magnitude, may enhance L-selectin expression (among other molecules) and, consequently, enhance the attachment potential of the embryo to uterine epithelium. In addition, the fluid flow in the uterus may stimulate the blastocyst to prepare for primary attachment through the L-selectin adhesion system. It is worth noting that the geometrical configuration of the trophoblasts of the blastocyst, as well as the size of the blastocyst may provide interesting differences between L-selectin shedding and potentially with L-selectin capping or spatial distribution in the presence of fluid flow. Further, definition of the intra-uterine fluid flow characteristics may be important in understanding blastocyst "mechanical" conditioning.

In conclusion, working with an optimized cell model for blastocyst/uterine epithelium interactions, consisting of trophoblast like Jeg-3 cells and uterine epithelial like Ishikawa cells, we have explored the effect of two exogenous influences on the L-selectin adhesion system which are of importance in the process of human implantation. These are the effect of the powerful glucocorticoid, dexamethasone, and that of hydrodynamic shear forces. Our results support the hypothesis that dexamethasone induces shedding of L-selectin in trophoblast like cells, however, an unexpected finding was the significant down regulation of MECA 79 reactive L-selectin ligands in the Ishikawa uterine epithelial cell line Both of these observations

would indicate that the use of dexamethasone in IVF protocols might in fact have a detrimental effect on the outcome.

Hydrodynamic shear forces generated by random fluid flow resulted in an ~30% upregulation of L-selectin on the Jeg-3 cells, but L-selectin shedding was negligible. Thus, treatment of an *in vitro* fertilized blastocyst with fluid flow, of a carefully determined range of magnitude, may enhance L-selectin expression (among other molecules) and, consequently, enhance the attachment potential of the embryo to uterine epithelium. This interesting concept will be explored in future work.

CHAPTER 5: QUANTIFYING ATTACHMENT STRENGTH

5.1 Introduction

Implantation begins with apposition, which involves *low bond strength* interactions between the blastocyst and the uterine surface epithelium [260]. In order for *structural* attachment to occur, that is, one which can resist shear forces due to fluid flow and other motions, there must first be an *initial interaction* that will slow the moving blastocyst and provide *primary attachment*. Recently, the presence of L-selectin on the human blastocyst, as well as a significant upregulation of the expression of the sulfated oligosaccharides that function as high-affinity counter-receptors for L-selectin on human uterine luminal and glandular epithelium during the window of receptivity has been demonstrated [141]. These initial interactions are thought to be mediated by L-selectin and its associated ligands. Secondary attachment is mediated at least in part by integrin/ligand interactions. The presence of integrin subunits on the human uterine endometrium throughout the menstrual cycle has been characterized through immunohistochemistry [120-122], and integrins have been known to be critical to the adhesion process in implantation since they were characterized by Lessey in the human endometrium in 1992. Of all the integrins which have been characterized, $\alpha_v \beta_3$ clearly stands out as the integrin most likely to be critical in the blastocyst attachment process, and it has been proposed as a potential receptor for embryonic attachment [364]. The $\alpha_v \beta_3$ dimer is simultaneously present in the uterine epithelium and in the trophoblast. It is thought that the anchorage of the embryo is made possible through “loop interactions”: thus, $\alpha_v \beta 3$ expressed on the trophoblast cells recognizes endometrial osteopontin, and

endometrial $\alpha_v \beta 3$ interacts with vitronectin and fibronectin expressed by the trophectoderm [132]. The αvβ3 integrin interacts specifically with the tripeptide sequence Arg-Gly-Asp (RGD), and this sequence has been implicated in trophoblast adhesion to extracellular matrix [13]. Thus, after initial interactions through the L-Selectin adhesion system, activation of the integrins and their ligands, through signaling cascades of molecules such as MAPK's and cytokines, results in *secondary adhesive interactions* which strengthen the primary bonds and provide firm attachment of the blastocyst to the uterine epithelium.

A parallel mechanism has been proposed between the steps involved in the human blastocyst implantation: embryo-endometrial apposition, attachment and invasion, and the leukocyte extravasation process: rolling/adhesion and invasion of the vascular endothelium [142]. These similarities introduced the possibility that the initial steps in implantation may include a rolling blastocyst which, in a manner similar to blood leukocytes, is slowed through the mechanism of low strength L-selectin-mediated primary bonding to the posterior wall of the uterus.

It has been demonstrated that the capture of leukocytes and subsequent rolling requires *critical thresholds* of shear stresses to occur [162-164]. The threshold shear stress range required to support rolling and attachment has been quantified at 0.4 to 1.9 dynes/cm^2 [166, 168]. This effect is not shared by the other selectins, but is unique to L-selectin. Furthermore, it appears to be an intrinsic property of the L-selectin molecule, as this dependence has been shown in cell-free immobilized L-selectin interacting with cell based or bead-immobilized L-selectin ligands [166, 167, 365].

Integrin-ligand interactions are known to have much stronger bonds. Depending on the concentration of the ligand (eg, fibronectin), bonding strengths ranging from 50 – 200 dynes/cm^2 have been measured in *in-vitro* systems [266, 269].

Many cellular adhesion measurement techniques exist, however, the differences between cellular adhesive phenomena in a dynamic versus a static environment have long been recognized, and for studying the initial processes involved in the capture and attachment of the blastocyst to the uterine epithelium, methods which incorporate flow (hydrodynamic shear) are the most appropriate type. Such techniques have been reported since the mid 1900's [261]. Today, several designs exist for the *quantification* of cellular adhesiveness through the use of hydrodynamic flow to produce shear (detachment) forces. These include the parallel plate flow apparatus [264], parallel disc viscometer [262], radial flow chambers [275], and the spinning disc detachment apparatus [366-368]. Each is described more fully in Chapter 1.

The parallel plate flow apparatus is ideally suited for observing and quantifying the process of leukocyte rolling, tethering and attachment due to the very low attachment forces which it can generate. Studies with induced shear stresses ranging from 0.3 to 35 dynes/cm^2 are the most common. Neutrophils have been shown to attach to L-selectin substrates with strengths of 1-3 dynes/cm^2 [166]. The surface expression of adhesion molecules such as ICAM-1 [284], VCAM-1 [285], E-selectin[286], P-selectin [287], L-selectin and PNAd [288], $\alpha_4\beta_1$ [289] and PSGL-1 [290] and other L-selectin ligands expressing sialyl Lewis *x* epitope [291] have all been characterized in this manner.

Typical parallel plate designs allow studies of cellular interactions under laminar flow on functionalized surfaces from the top-down, or sometimes, a side view with microscopic observation and analysis. A novel modification of typical parallel plate flow chamber designs that has been incorporated in the apparatus used in this work is a Quartz Crystal Microbalance (QCM) sensor which responds to changes in the mass of a coating on its surface with a well defined resonant frequency shift [369, 370], thus allowing real time monitoring of cell detachment kinetics. The shift in frequency can be correlated with quantitative changes in cell population on the QCM surface allowing the generation of detachment strengths profiles of L-selectin/ligand binding. This sensor [369], and its response characteristics [371] are shown in figure 33.

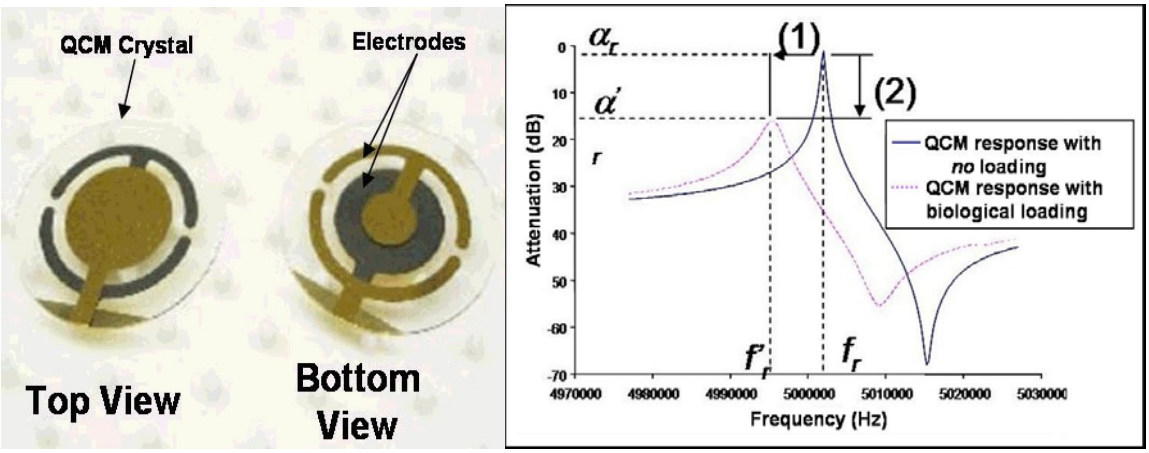


Figure 33: (A) QCM Sensor [371];
(B) Typical frequency response of a quartz crystal resonator [369].

Radial flow chambers, and the spinning disc detachment apparatus have been used extensively to quantify adhesion interactions with integrin and their ligands. The radial

flow chamber (RFC) is a dynamic cell detachment technique which can generate a spatially dependant range of shear stresses simultaneously. Radial flow chambers utilize axisymmetric radial flow to produce a shear stress that *decreases* with *increasing radial position* [271], however, a significant disadvantage with this system, is the complexity of the hydrodynamic detachment forces at relatively small radial distances from the inlet. The spinning disc cell detachment apparatus, based on the same design principle as the parallel disc viscometer [262], is a more recently developed instrument [366]. The parallel disc viscometer was one of the very early designs for cellular adhesion measurements, adapted for use in a biological system from instruments developed for mechanical engineering applications[262]. The spinning disc design consists of a single bottom plate where, similarly to the radial flow chamber, a range of shear forces are generated simultaneously by the chamber fluid as it undergoes laminar flow, in a radial pattern over the disc. Of these three hydrodynamic systems, the spinning disc is the only configuration that applies a linear range of detachment forces under uniform and constant chemical conditions at the surface [256]. Applications of the spinning disc CDA include: red cell adhesion to Teflon®, glass and polyethylene [270], adhesion of 3T3 cells (immortalized fibroblast cell line from Swiss mouse embryo tissue) to a chemically homologous series of copolymers based on hydroxyethylmethacrylate (HEMA) and ethylmethacrylate (EMA) [265], and initial integrin-fibronectin interactions using K562 cells [266].

Substrates for studies of the interactions of cells with protein ligands or components of the extracellular matrix are often prepared by allowing a protein to adsorb from solution

onto a glass or polymer surface. This method is effective for many studies, however, it can fall short in cases where reproducible, well defined and carefully controlled protein coating is required. When the material to be functionalized is gold, this requirement can be particularly challenging. Self assembled monolayers, formed by the spontaneous ordering of terminally functionalized alkanethiols onto the gold provide a convenient method for tailoring substrates with ligands, proteins and other groups in a highly controlled manner [372].

The structure of thiol terminated SAM's on gold is well established [373]. The sulfur atoms coordinate to the threefold sites of the gold (111) surface to give a closed packed array of alkyl chains, which is particularly efficient where n >10 for the n-alkanethiols used. The strong interactions between the sulfur and the gold substrate contributes substantially to the overall adsorption energy, which is typically in the range of 35-45 kcal/mole. The thiols are believed to attach primarily to the threefold hollow sites of the gold surface, losing the proton in the process, and forming a slightly tilted, highly crystalline overlayer configuration as shown in figure 34 [374].

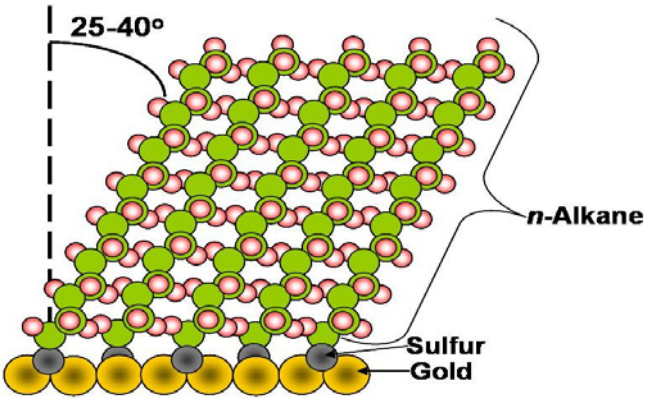


Figure 34: Structure of n-alkanethiol self assembled monolayer adsorbed on gold: molecules assemble in slightly tilted, all trans configuration in order to optimize lateral interactions between molecules within the monolayer (adapted from[374]).

Proteins may then be adsorbed onto the SAM, or they may be covalently attached. Furthermore, when precise control of the protein density, is desired, *mixed monolayers* may be utilized. Mixed monolayers, consist of only a percentage of the terminal groups which are reactive, and may serve to maximize accessibility and minimize steric hindrance for the receptor. They are highly effective for this application, since it has been shown that multicomponent monolayers *do not segregate* into discrete single-component domains [375]. Mixed SAM's are produced on gold substrates from solutions of two or more alkanethiols, where one component (**X**) contains an inert terminal group (eg, CH_3) and a second component (**Y)** contains a reactive terminal functional group (eg, COOH), which determines the reactivity of the SAM. To preserve crystallinity, and maximize accessibility, the relative chain lengths of X and Y should be close, with X < Y. An ideal mixed monolayer system would thus consist of $(HS\text{-}(CH_2)_{16}\text{-}OH)$ /$(HS\text{-}(CH_2)_n\text{-}CH_3)$ where n = 11-15 [374, 375].

It is possible to covalently attach a biomolecule (such as a ligand binding protein) onto a SAM modified gold surface. Several immobilization strategies are available, however, carboxyl terminated (-COOH) alkanethiols are recommended, as this approach avoids the possibility of protein-protein cross linking [374]. The carboxyl group can then be activated in a carbodiimide (N=C=N) reaction where it will undergo nucleophilic substitution in the presence of a primary amine, as shown below. In protein molecules, the primary amine may be a surface exposed lysine, which is common due to the hydrophilic nature of lysines, which insures that they predominate on protein surfaces.

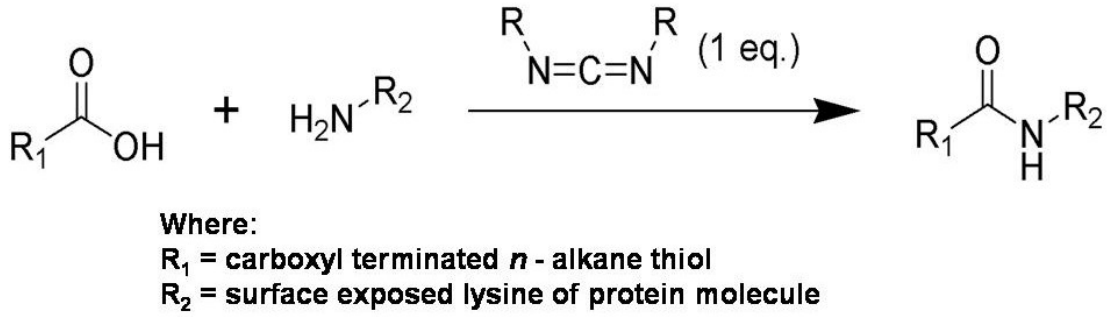


Figure 35: Covalent coupling of protein molecule to carboxyl terminated SAM through carbodiimide reaction [376]

Attachment strengths for both the L-selectin and integrin adhesion systems have not been quantified with regard to trophoblast-uterine epithelial interactions. It is the ultimate goal of project to quantify the attachment strengths of the protein/ligand interactions as a function of expression which may provide compelling evidence toward *critical* levels of L-selectin/L-selectin ligand, as well as integrin/ligand expression which would be necessary to achieve *primary attachment*, and strengthen this attachment through *secondary interactions* which ultimately facilitate implantation. The first step toward this goal is the work reported herein. That is, evaluation attachment strengths of the primary and secondary adhesion mechanisms in the optimized trophoblast-uterine epithelial cell model consisting of Jeg-3 and Ishikawa cell lines through the use of appropriate cell detachment instrumentation, and well defined substrates.

5.2 Materials and Methods

Antibodies and Antigens

For deposition of the SAM on all gold substrates, 11-Mercaptoundecanoic Acid (MUA), 1-Decanethiol (NDt), (N-3- Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-Hydroxysuccinimide (NHS), and ethanolamine were all obtained from Sigma Aldrich *(St. Louis, MO)*. All organic compounds were of the highest purity available, and were used without further purification.). Fibronectin was also purchased from Sigma Aldrich, and was used for coating the spinning disc at a concentration of 5 ug/ml in PBS. L-Selectin Fc Chimera was obtained from R&D Systems,(*San Diego, CA*), and was used at a concentration of 25 ug/ml in PBS when used in the coating for the spinning disc. LEAF purified anti-human CD62L (L-Selectin) (DREG-56) was obtained from *BioLegend* (*San Diego, CA*). Phenol red free culture medium, D-MEM F-12 was purchased from Gibco/Invitrogen #2104 (*Carlsbad, CA).*

Preparation of Ishikawa Cells for Detachment Assays

Ishikawa cells were passaged and incubated for at least 24h under normal conditions in order to assure log-growth properties. Cells were harvested for testing during the log-growth period; ~24 to 72 hours after passaging. Cells were first rinsed in PBS, trypsinized for 7-10 minutes at 37^{o}C, then centrifuged and resuspended in serum free phenol red free culture medium at a concentration of 6 x 10^{5} cells/ml. Cells were incubated at 37^{o}C in suspension until used.

Parallel Plate Flow Apparatus

The parallel plate flow cell detachment apparatus has a defined flow profile that closely approximates the hydrodynamic features found in the vasculature [377]. This apparatus is ideally suited for observing and quantifying the process of leukocyte rolling, tethering and attachment due to the *very low* attachment forces which it can generate. Studies with functionalized surfaces with induced shear stresses ranging from 0.3 to 35 dynes/cm^2 are the most common. [166, 281, 282]. The apparatus used in this work was designed, manufactured, and validated in our lab [378] and is similar in design and function to many of the commercially available parallel plate flow chambers on the market today. It is capable of generating hydrodynamic shear forces in the range of 0 – 10 dynes/cm^2, through laminar flow generated by a peristaltic pump (Harvard Apparatus - Peristaltic Pump 77) selected for its accuracy (+/- 1%; similar to that of a syringe pump) and ability to pump very high volumes of fluid. A reservoir system was implemented to achieve precision control of flow rate and induced shear rates *with minimal pulsation*. Figure 11 (Chapter 1) shows an exploded schematic of the flow chamber, with the laminar flow profile that occurs over the functionalized surface. The laminar flow rate and applied wall shear stress are linearly proportional through equation (7):

The wall shear stress, τ_{wall}, can be calculated from the momentum balance for a Newtonian fluid [283], and is given by:

$$\tau_{wall} = \frac{3\mu Q}{2h^2 b} \qquad (7)$$

where μ= Coefficient of viscosity, Q = volumetric flow rate, 2h = channel height and b = channel width [164]. The flow chamber is shown in figure 36 A. For calculating the flow rate the critical dimensions, are defined by the gasket plate (Figure 36B) .

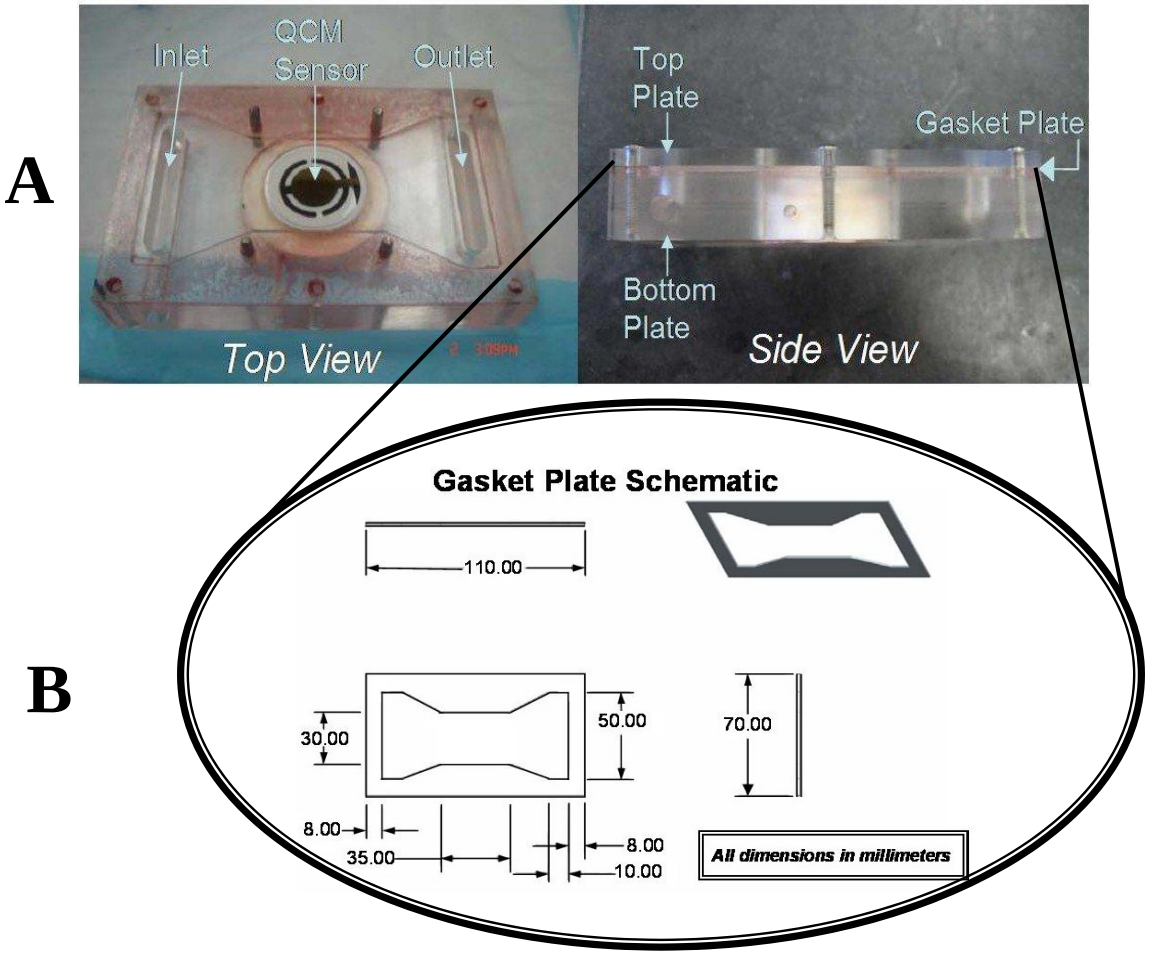


Figure 36: (A) Parallel Plate Flow Apparatus for Ishikawa Cell Detachment Assay (B) Gasket Plate Schematic [378]

A unique modification to the typical flow chamber design, which was validated in this work, is the addition of a quartz crystal microbalance (QCM) as a detection device (figure 32A). A QCM is a piezoelectric sensor which works by deforming at a resonant frequency when a voltage is applied. Mass deposited on or removed from the surface will cause a change in the frequency at which the crystal resonates [369]. Background stability of the sensor output in the presence of temperature and flow variations was monitored, however, absolute quantification of the cell detachment levels was made by microscopic observation.

The gold surface of the QCM was functionalized with L-selectin, covalently bound to the substrate through a mixed-alkane thiol SAM "linker", as described in the following section. PBS at 37 ^{o}C was first flowed through the system for a period of approximately 10 minutes to achieve a baseline reading. Ishikawa cells were prepared for testing by brief trypsinization to detach them from the culture flask, after which they were placed in a suspension of serum-free culture medium and incubated at 37^{o}C during the baseline equilibration. They were then added to the system and allowed to adhere, under static conditions, for approximately 5 minutes, at 37^{o}C. The cell density was observed microscopically, and recorded. Flow was then applied to the system at known shear force for 5 minutes and the cell density was recorded again. `The shear force which resulted in a 50% detachment of the cells was that which was taken to represent the cell detachment strength related to L-selectin/L-selectin ligand bonds [256]. For the control experiment, an L-selectin antibody (DREG-56) was applied to the L-selectin coated sensor to block binding sites.

Deposition of Mixed SAM on Gold

Gold substrates were first cleaned in a solution of 30% H_2O_2, 25% NH_3, H_2O (1:15) at 80^oC for 5-10 minutes. Cleaned substrates were immersed in a binary mixture of 2.5 mM MUA and 7.5 mM NDt in absolute ethanol for three (3) hours at RT, followed by a thorough rinse in absolute ethanol, after which they were allowed to dry in a flow of clean dry air. A monolayer coating of 90Å thickness was measured by elipsometry.

Covalent Attachment of Protein to SAM

The SAM coated gold substrate was immersed in a solution of 20mM NHS and 10 mM EDC in ultrapure water for 2 hours at room temperature (RT) for "activation", followed by immersion in a solution of 25 ug/ml L-selectin Fc Chimera in 10 mM PBS at pH 7.4 for 2 hours at RT. Remaining active binding sites were then blocked with 1M ethanolamine at pH 9.0 at RT for 20 minutes. Following a thorough rinsing of the substrate in ultrapure water, all remaining binding sites are blocked with 1% BSA in 10 mM PBS for two hours at RT. A detailed protocol for SAM deposition and the covalent attachment of L-selectin can be found in Appendix G.

Spinning Disc Apparatus

Figure 37 shows the spinning disc apparatus used in this experimentation.

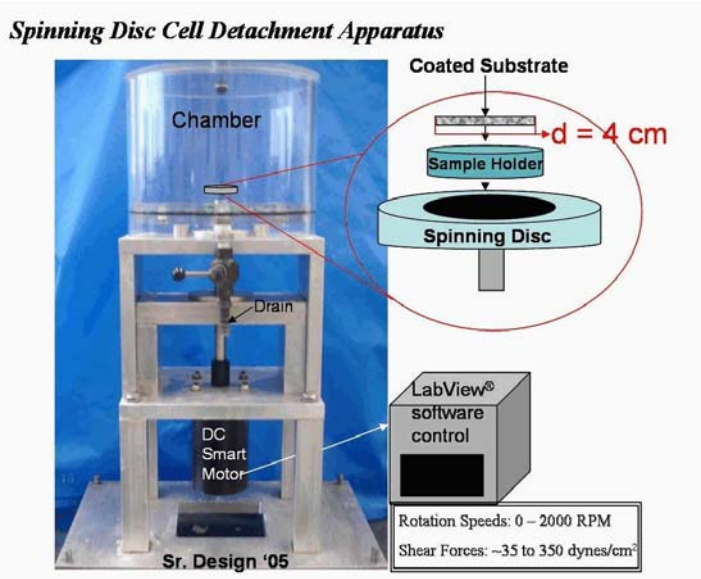


Figure 37: Spinning Disc Cell Detachment Apparatus(SDCDA)

The hydrodynamic shear detachment stresses that can be accurately generated with this (validated) spinning disc cell detachment apparatus range from ~15 to 350 dynes/cm^2 . The apparatus was built with specifications which would assure the achievement of laminar flow. For a given rotational speed, the velocity and temperature of the fluid as well as the boundary layer thickness, δ, are constant, when the radius of the fluid chamber, R, is infinite compared to the radius of the spinning disc, r. The infinite disc approximation is valid when $R/\delta > 50$.

Electrochemical Validation

To test this criterion an electrochemical method described by Garcia [248] was conducted utilizing the redox couple ferrocyanide/ferricyanide . The electrochemical solution was prepared by dissolving 1.0 M KCl, 0.01 M $K_4Fe(CN)_6$, and 0.01 M $K_3Fe(CN)_6$ (generous

gifts of Drexel University Chemistry Department), in DI water. KCl was used as the common ion to suppress electrical migration effects. Electrochemical measurements were performed with a l5 mm diameter disc of platinum foil glued onto the sample holder. The electrical circuit was established with an insulated wire soldered to the underside of the platinum disc, inserted through the shaft and connected to a slip ring assembly, which was then connected to a potentiostat (model #283, EG&G, Princeton, NJ). The counter-electrodes consisted of two strips of platinum foil (each 2"L x 0.5"W) placed against the interior chamber wall on opposite sides of the chamber. A saturated calomel electrode (SCE) (Drexel University, Chemistry Department) was used as the reference electrode (figure 38). It was placed centrally in the chamber, at a small vertical distance from the platinum disc to minimize IR drop. At several disc speeds, single voltage sweeps over a potential range of + 0.4V to -1.0V were made, and the resulting current was recorded

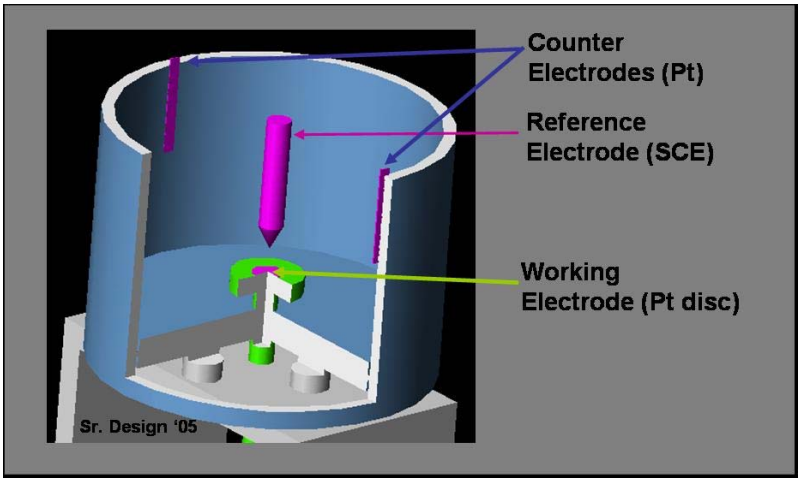


Figure 38: Electrode configuration for spinning disc electrochemical validation of flow characteristics [379]

Experimental Protocol

The Ishikawa cells were prepared for testing by detaching them from the culture flask by brief trypsinization, after which the cell suspension was centrifuged, and the cells were re-suspended in serum free culture medium, and counted. The functionalized disc was masked with a custom cut silicone rubber sheet containing a centrally located 3.5cm x 1cm opening . The cells were seeded onto the disc at a concentration 6×10^5 cells/ml and allowed to adhere, under static conditions, for 15 minutes, at 37^oC. The silicone rubber mask was removed, the disc was placed on the sample holder in the chamber, and the chamber was carefully filled with 37^oC PBS, while shielding them from any turbulence. Each spinning disc experiment was 5 minutes in duration. The 2320D Smart Motor (Animatics, CA) was controlled by LabView software which enabled input of a desired velocity (rpm) and rate of acceleration and deceleration.
At the conclusion of the test, the PBS was drained, the mask was precisely replaced over the functionalized area with the remaining attached cells, and the cells were counted as a function of distance from the center. Images of the cell population were taken in 2mm increments across the diameter of the disc, and the cells were manually counted in each image. Figure 39 shows the experimental setup for quantification of the results.

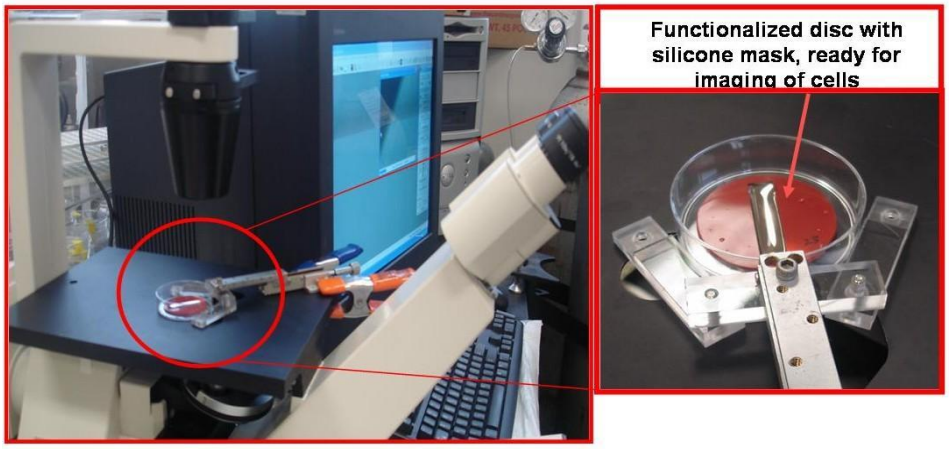


Figure 39: Experimental setup for microscopic imaging of the disc with attached cells, and the modified micrometer used to move the disc in precise 2 mm increments.

5.3 Results

Parallel Plate Flow Apparatus

The parallel plate flow apparatus was used to measure (low) detachment strengths associated with the L-selectin adhesion system. Validation of the QCM sensor, for use in the parallel plate flow apparatus, first required the evaluation of signal stability in the presence of flow, as shown in figure 40.

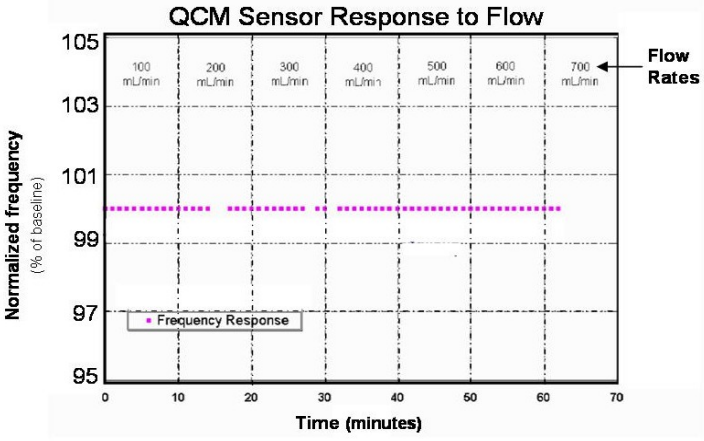


Figure 40: QCM response to flow, over the hydrodynamic shear range of 0 to 10 dynes/cm^2

The parallel plate flow apparatus was then used to examine the detachment strength of Ishikawa cells to substrate functionalized with L-selectin covalently bonded to an SAM. The detachment strength is determined by the shear force which is required to detach 50% of the cells. Figure 41 shows the results of this test. Cells were counted at seeding and after the 5 minute test.

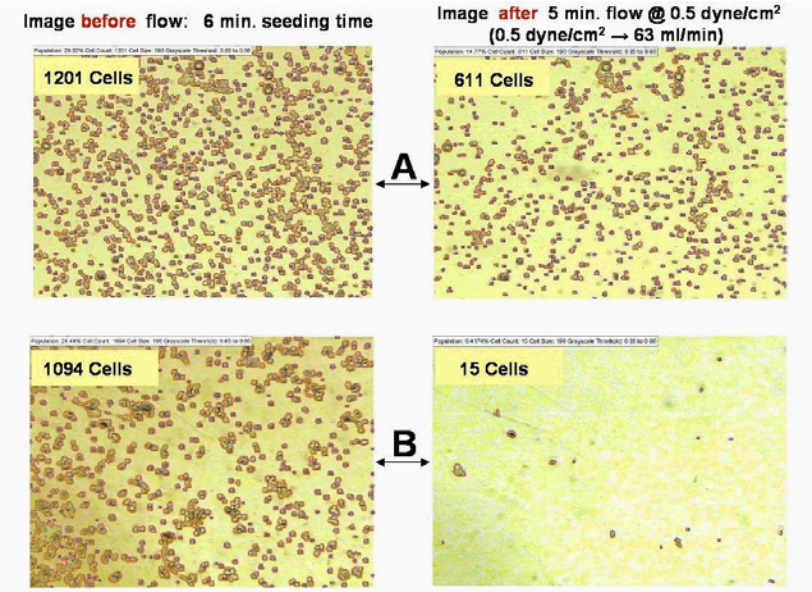


Figure 41: Parallel Plate cell detachment assay using Ishikawa cells:
(A) Ishikawa cells on L-selectin functionalized gold surface
(B) Ishikawa cells on DREG 56 antibody blocked L-selectin functionalized gold surface

During the cell detachment assay, the frequency response of the QCM sensor was monitored as well (figure 42)

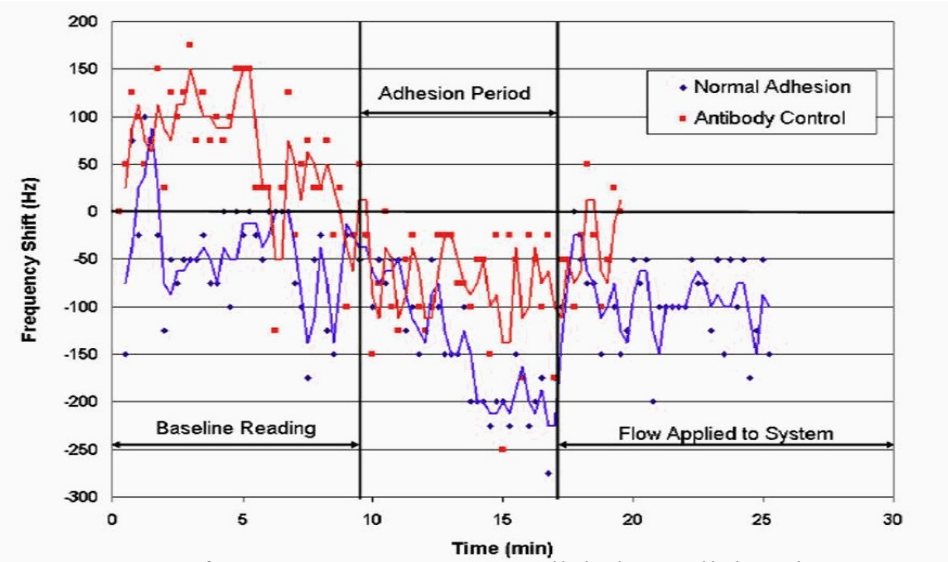


Figure 42: QCM frequency response in parallel plate cell detachment assays using Ishikawa cells on L-selectin functionalized sensor

Spinning Disc Cell Detachment Apparatus

The spinning disc cell detachment apparatus was used to evaluate (stronger, secondary) detachment strengths associated with the integrin/ligand adhesion system. Prior to the experimental work the theoretical flow pattern of the system was validated through an electrochemical validation protocol using the ferrocyanide/ferricyanide redox couple, as shown in Figure 43.

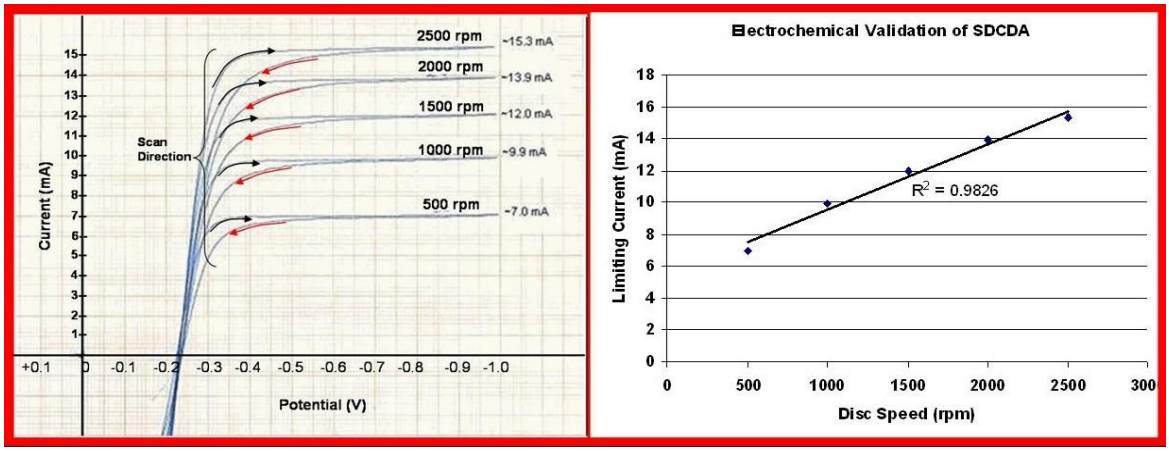


Figure 43: (A) Current-Voltage curves showing limiting current for the spinning disc electrode as a function of disc speed.
(B) The values for the limiting current obtained from the polarization curves, versus disc speed.

The spinning disc rotational speeds used for this testing ranged from 1500 to 2000 RPM. The detachment profile for cells at these two different angular velocities was evaluated for consistency. Figure 44 shows the Ishikawa cell detachment profile on a 100% fibronectin coated substrate for 1500 and 2000 RPM, and the resultant adhesion strength. Cell counts were normalized to the number of adherent cells at the disc center.

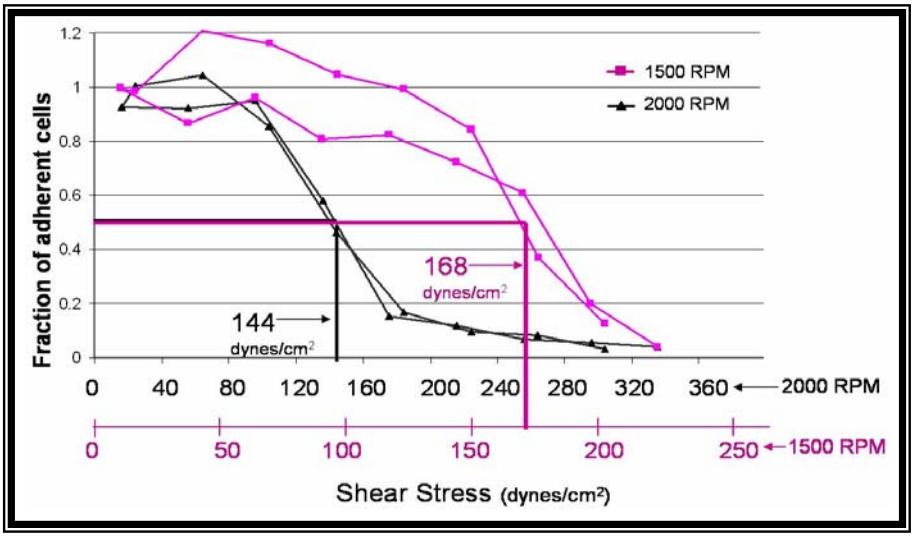
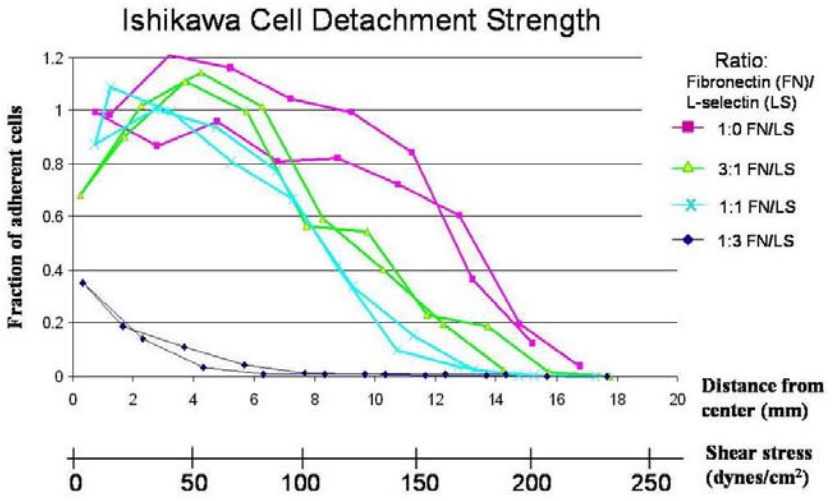


Figure 44: Determination of detachment strength for Ishikawa cells on a fibronectin substrate at two different angular velocities.

Attachment strengths attributed to integrin/ligand interactions, were investigated with the spinning disc apparatus. The potential modulating effect combining L-selectin and fibronectin was also explored by quantifying attachment strengths of Ishikawa cells on coatings of various ratios of fibronectin (FN) and L-Selectin (LS). Figure 45 shows the detachment profile for Ishikawa cells on various substrates, as well as the relationship of these values to the % of fibronectin.

A



B

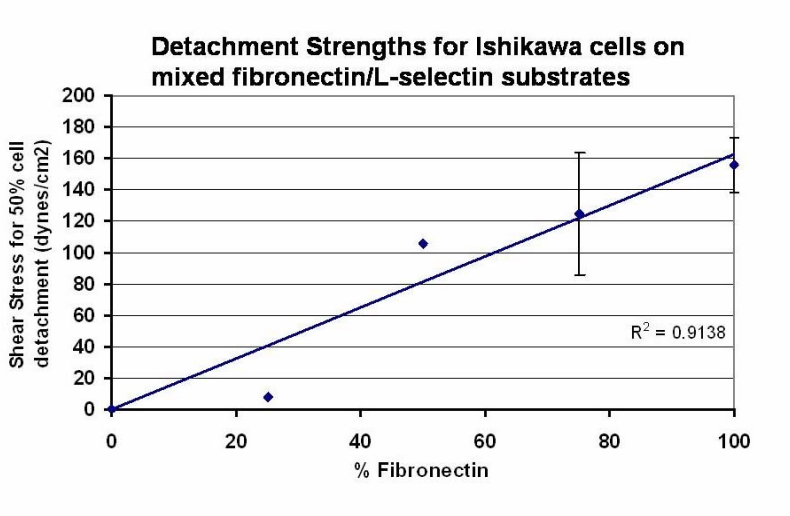


Figure 45: (A) Cell detachment profile for Ishikawa cells on mixtures of fibronectin and L-selectin of varying ratios
(B) Relationship of cell detachment strength to fibronectin concentration

5.4 Discussion

Parallel Plate Flow Apparatus

While parallel plate flow chambers are useful for monitoring "low strength" primary cellular interactions, one disadvantage is that most flow chamber designs must utilize

costly high-speed cameras to observe cell motion and velocity, however typically this is not done in real-time. Experimentation with high-speed cameras requires the user to review collected data and conduct secondary analysis to characterize cellular interactions. Computer programs do exist to calculate cell numbers when recorded through a microscope however, they are cumbersome to use, and can be inaccurate especially when high density cell populations are used. The parallel plate flow chamber with a QCM sensor incorporated is a novel configuration that will allow the monitoring of cell detachment kinetics in real time. The work reported in this thesis is the first step towards the goal of real time monitoring, without microscopic observation and cell counting. The QCM sensor was first evaluated for its suitability in this application by examining background sensitivities, such as frequency shifts in the presence of hydrodynamic flow. The high level of signal stability that was seen with the QCM sensor (Figure 40), over all flow rates that are used in this apparatus, verifies that this sensor has little to no sensitivity to hydrodynamic flow, and thus is suitable for use in this application. Preliminary work to determine the optimum incubation period for the cells to attach to the L-Selectin surface showed that it should not exceed 6 minutes. After this, the detachment strength of the cells exceeded the maximum shear forces (10 dynes/cm^2) that could be generated by this apparatus [378]. This increase in detachment strength is thought to be associated with cell spreading, which occurs after the strength is thought to be associated with cell spreading, which occurs after the preliminary attachment processes associated with the L-selectin adhesion system .

When the Ishikawa cells were added and incubated statically for 6 minutes, the shear stress that resulted in the detachment of 50 % of the cells was 0.5 dynes/cm^2 (flow rate = 63 ml/min). The threshold shear stress range required to support rolling and attachment in neutrophils through L-selectin/L-selectin ligand interactions has been quantified at 0.4 to 1.9 dynes/cm^2 [166, 168]. The detachment strength determined here correlates very well with these values. When the L-selectin coating was blocked by DREG-56, an L-selectin antibody, virtually all of the Ishikawa cells detached under the same shear stress. This supports the conclusion that the detachment forces being measured are due to L-selectin/L-selectin ligand interactions.

Though these tests relied greatly on cell counting for final quantification, the output of the QCM sensor correlated well with the temporal detachment process. In figure 42, QCM signals are shown for two samples. In one test, represented by a blue line, the adhesive interactions of Ishikawa cells on an L-selectin functionalized surface were monitored (normal adhesion), and in the other test, represented by a red line, the adhesive interactions of Ishikawa cells on a surface, functionalized with L-selectin, but blocked by an L-selectin antibody, were monitored (anti-body control). For the first 10 minutes, baseline stability readings were taken. This is followed by the perfusion of Ishikawa cells into the chamber and a 6 minute static incubation period. The third section represents the sensor signals while the hydrodynamic shear forces are being applied.

The baseline readings for both sensors, while somewhat noisy, do not show any significant level of net drift in the signal. However, the static incubation period shows

considerable between the "control" and the "normal adhesion" samples. The steady downward drift of the "normal adhesion" signal can be interpreted as the cells adhering to the L-selectin as they contact functionalized surface, thus adding mass to the sensor, and dampening the frequency of the crystal resonator. In the control sensor, even though a slight change in the signal is seen, there is no downward drift indicating that adhesion to the surface probably not occurring. Upon the initiation of the flow, the signal for each sensor increases however, by different amounts. Whereas the control sensor returns to its original signal at the end of the stability period, the test sensor (normal adhesion) returns to a signal that is ~50 db lower than its value at the end of the stability testing. Both of these observations are consistent with the microscopically determined results. That is, ~50% of the cells remained attached to the L-selectin functionalized surface, after the hydrodynamic shear stresses were introduced, thus the remaining cell mass was indicated by the sensor signal. However, with the antibody blocked control sensor, virtually all cells were removed from the surface under hydrodynamic forces, so the sensor signal returned to its original signal before cells were added. In conclusion, though the data are not quantitative, we have obtained positive results to support the feasibility of incorporating a QCM sensor into this device as a means to monitor in real time, the cellular adhesion events associated with "low strength" primary attachment mechanisms such as those which occur between L-selectin and its ligands. However, further validation of this modified apparatus is required to obtain direct quantitative information. Furthermore, by visually quantifying the cellular attachment characteristics, we have, for

the first time, determined an attachment strength value for the L-selectin/L-selectin ligand adhesion system in a cell model for the process of blastocyst implantation.

Spinning Disc Cell Detachment Apparatus (CDA)

The spinning disc apparatus generates shear forces through laminar flow, in a radial pattern over the disc. As the disc rotates, the fluid is drawn in axially from the surroundings, and as it approaches the surface of the disc, it acquires a rotational motion which forces it to exit radially [248]. A schematic of the idealized flow pattern for the spinning disc apparatus is shown in figure 11: Chapter 1 [265]. To achieve laminar flow, the surface must be uniformly accessible, and the diffusive field must be uniform across the surface of the disc [248].

The spinning disc cell detachment apparatus was built with specifications so that laminar flow could be achieved (e.g. no head space in the filled chamber, chamber dimensions infinitely large compared to the disc size). For steady laminar flow, the velocity, temperature and concentration boundary layer thicknesses are constant for a given rotational speed and independent of radial distance [248]. Under these conditions, the disc is a uniformly accessible surface and the diffusive field is uniform over the surface of the disc. In this design, the shear stress, τ, varies linearly with radial distance and is given by:

$$\tau = 0.800\ r\ (\rho\mu\omega^3)^{1/2} \qquad (4)$$

Where ρ = fluid density, μ = fluid viscosity and ω = angular velocity.

Electrochemical validation of the spinning disc CDA flow pattern was performed in accordance with the protocol outlined by Garcia [248]. On a platinum electrode, the reduction of ferricyanide to ferrocyanide is a rapid charge transfer reaction. The overall reaction rate is therefore dependent *only* on the transport of ions. This transport is limited by diffusion, which is influenced by three factors: migration due to an electrical gradient, diffusion due to a concentration gradient, and convection as a result of bulk fluid movement. The effects of the electrical gradient (IR drop) are negated with the use of a common ion (KCl) at a high concentration, (1M). Therefore for an instantaneous reaction at the electrode, the reaction rate is dependent on diffusion and convection. The flow of ions to the platinum working electrode increases as the potential applied to the system increases, and this is seen by an increase in current, until a diffusion limited current density is obtained [248, 380, 381]. The limiting current then remains constant with further increases in potential, unless another cathodic reaction (such as the reduction of water) is activated. If a uniform diffusive field exists across the disc surface then the current density achieved at a given voltage *and angular velocity* should be according to the equation:

$$i_L = \frac{nFc_i D^{2/3} \omega^{1/2}}{1.61 \upsilon^{1/6}} \qquad (7)$$

where iL = limiting current density, n = number of electrons in the charge transfer reaction, F = Faraday's constant, c_i = ion concentration, D = diffusivity, ω = angular velocity and υ = kinematic viscosity [248]. Figure 42 (A) shows the current- voltage

curves obtained with this spinning disc apparatus, where the limiting current is directly influenced by the angular velocity of the rotating disc. Figure 43(B) shows that this relationship is linear, in agreement with the theory presented, thus for the speeds examined, the transport patterns generated correlate well with those for an infinite spinning disc.

In exploring integrin/ligand interactions, with the spinning disc, the Ishikawa uterine epithelial cell line was used, with substrates functionalized with the integrin receptor, fibronectin. Fibronectin is a ligand which can bind to several integrin receptors, including $\alpha_3\beta_1$, $\alpha_8\beta_1$and $\alpha v\beta_3$ [382]. There is substantial evidence that the integrin $\alpha_v\beta_3$ is directly involved in the blastocyst/uterine epithelial adhesion process. This integrin binds to the RGD motif in fibronectin, osteopontin, vitronectin and laminin, among others [125, 126]. It is also expressed in Ishikawa cells [81].

Prior to more in-depth probing of the integrin/ligand interactions with Ishikawa cells, we wished to answer one additional question with regard the spinning disc validation regarding the consistency of the range of forces generated at different angular velocities. Theoretically, the detachment strengths for the same cell line adhering to the same substrate under the same conditions should be the same regardless of the angular velocity at which it was tested, with the radial distance at which 50% of the cells detach being the only variable. In figure 44 the results of the detachment experiments at different angular speeds for Ishikawa cells on 100 % fibronectin are shown, where the normalized fraction of adherent cells is plotted versus the shear stress. For a cell population with normally distributed adhesion properties, the detachment profile is predicted to fit a symmetrical

logistic equation [248]. The detachment strength values (τ_{50}) were determined using the following relationship:

$$f = \frac{f_0}{1 + \exp\left[b\left(\tau - \tau_{50}\right)\right]} \qquad (8)$$

where f is the experimental adherent cell fraction, τ is the shear stress on the disc surface, f_0 is the cell fraction at "0" stress (typically near 1), b is the decay slope, and τ_{50} is the inflection point, ideally where f=0.5 The τ_{50} values for obtained (144 and 168 dynes/cm^2 for 2000 and 1500 RPM, respectively) are in good agreement with each other(σ = 17), Though no data has been reported regarding quantification of integrin/ligand interactions with Ishikawa cells, the values obtained here are within the range of normal adhesive strengths for these systems i.e. 50-200 dynes/cm^2 [266, 269]. Though optimum experimental protocol would specify all tests at the same speed, the results of this examination support direct comparison of test data obtained at different speeds. The small variations seen are hypothesized to be a result in heterogeneities in the cell populations, cell size and possibly receptor numbers, since the cells used in a series of tests were detached and counted at the same time, but incubated in a centrifuge tube (where they could not reattach) for varying amounts of time prior to seeding on the functionalized disc. These could be minimized by protocol revisions for cell preparation.

After the initial low strength binding interactions mediated through the L-selectin adhesion system, integrins are thought to play an important role in affecting secondary

strengthening interactions. In parallel to the temporal adhesion interactions demonstrated in neutrophils, primary low strength L-selectin mediated interactions in the process of blastocyst implantation are thought to initiate the up-regulation and/or activation of integrins through MAPK signaling cascades. We questioned whether or not the presence of L-selectin in the substrate would facilitate this sequential series of interactions, and therefore influence the resulting detachment strength values. Thus, attachment strengths of Ishikawa cells on coatings of various ratios of fibronectin (FN) and L-Selectin (LS) were evaluated. The ratios used were 25% fibronectin/75% L-selectin (1:3 FN/LS), 50% of each component (1:1 FN/LS), 75% fibronectin/25% L-selectin (3:1 FN/LS), and 100 % fibronectin (1:0 FN/LS). In figure 45A, the fraction of adhered cells is plotted versus shear stress, and radial distance from the center for each of these conditions. The progressive increase in τ_{50} with fibronectin concentration is evident, however, this linear relationship is clearly seen in figure 45B.

Garcia, et al. has used the spinning disc apparatus to elucidate cooperative binding effects with integrin/ligand adhesion interactions [266, 269]) in untreated IMR-90 (human fibroblast) cells. However, it was found that, with short incubation times (15 minutes) these effects were absent. Instead, it was determined that a linear relationship existed between the adhesion strength and fibronectin density. The results obtained with Ishikawa cells would also support this conclusion. The optimum incubation time in this testing was determined to be 15 minutes. At longer incubation times, the cells were observed to be spreading, and had attachment strength increased beyond values which could be generated by the spinning apparatus. Both of these observations would indicate

that more complex interactions between the cells and substrate were occurring after 15 minutes.

In conclusion, adhesive interactions between Ishikawa cells and L-selectin have been quantified through the use of a parallel plate flow chamber, and found to be in the range of values obtained for similar interactions with neutrophils. Furthermore, integrin/ligand interactions with Ishikawa cells have been quantified with a spinning disc cell detachment apparatus, and been found to be within the range of normal adhesive strengths for these systems i.e. 50-200 dynes/cm^2. Experimental results with the mixed L-selectin/fibronectin substrates support the hypothesis that within the 15 minute incubation time, L-Selectin mediated integrin activation is probably not occurring, and the various adhesion strengths seen are most likely due to the range of fibronectin densities in the substrate.

CHAPTER 6: SUMMARY AND CONCLUSIONS

This project has focused on furthering the understanding of biochemical and mechanical mechanisms involved in the human blastocyst implantation process, with the long term goal of developing strategies for enhancing the *initial* embryonic attachment event in women undergoing fertility treatments. This occurrence is thought to be mediated by the L-selectin adhesion system. Further secondary events, namely strengthening of the adhesive bond, are thought to occur through integrin/ligand interactions. Little information exists, however, as to the effects of cytokines, exogenous hormones, and other endocrine mediators on these critical events. Further, no information exists with regard to the mechanical aspects of the attachment process.

Toward that end, a cell model, consisting of trophoblast-like Jeg-3 cells and uterine epithelial like Ishikawa cells has been established. *The Jeg-3 cell line has been shown to constituitively express L-selectin, and undergo modulation of the expression levels in response to progesterone and estrogen hormonal influences*. Most importantly, the L-selectin is expressed on the cell membrane, a required condition to facilitate adhesive interactions with L-selectin ligands. The other trophoblast like cell lines (JAR and BeWo) which were evaluated did not appear to express this protein on the cell membrane.

Ishikawa (uterine epithelial) cells have been well characterized with regard to the expression of protein biomarkers of uterine receptivity, however, they have not been evaluated for expression of L-selectin ligands. We found that Ishikawa cells do express

MECA 79 reactive L-selectin ligands, which contain the requisite sulfated sialyl Lewis x oligosaccharide. Further, we found that ligands expressed at MW 80 and 90 kDa are responsive to hormonal conditioning by progesterone and estrogen. One specific L-selectin ligand, MadCAM was also identified. Since trophoblast attachment and implantation into uterine epithelium has been proposed to mimic leukocyte attachment and extravasation through the vascular endothelium [142] we hypothesized that Ishikawa cells would only express the 40 kDa glycoform of MadCAM-1 analogous to that found in vascular endothelium. Confirming this hypothesis, a strong signal was detected using an antibody to the 40 kDa glycoform of MadCAM-1 by immunoblot analysis, while an antibody against the 60kDa glycoform did not react. *In this work, the constitutive expression, and hormonal regulation of the class of MECA 79 reactive L-selectin ligands, as well as MadCAM-1, is a novel and important finding which will allow this cell line to be utilized as a cell model in future experiments.*

Dexamethasone and other glucocorticoids such as prednisone, prednisolone and methyl prednisolone have been used to treat infertility as a routine step in IVF treatment protocols, and in the context of inflammatory disease, such as PCOS. The modulation of L-selectin/ligand system by glucocorticoids in the reproductive process is not well-understood. The Jeg-3/Ishikawa trophoblast/uterine epithelial cell model was used to explore the effect of physiological levels of dexamethasone on the L-selectin adhesion system. When we evaluated the effect of dexamethasone on cellular L-selectin expression by western blot, we found a slight upregulation after 48 hours. When the culture medium was analyzed for soluble L-selectin by ELISA, it was determined that

dexamethasone induces shedding of L-selectin in trophoblast like cells, an effect which has been characterized in neutrophils. However, an unexpected finding was the significant *down regulation of MECA 79 reactive L-selectin ligands in the Ishikawa uterine epithelial cell line, as determined by western blot.* Both of these observations are novel and important findings which indicate that the use of dexamethasone in IVF protocols might in fact have a detrimental effect on the outcome.

Intra-uterine fluid flow characteristics are not well understood, however, it can be assumed that there is at least a mixing flow provided by asynchronous contractions [176] and mucin secretions by the endometrium [141]. Fluid flow has been shown to increase L-selectin expression in neutrophils [162]. We tested the condition of hydrodynamic fluid flow on the Jeg-3 trophoblast-like cell line to determine its potential modulating effect on the expression of L-selectin, and found an increase in L-selectin expression of around 30%. Though L-selectin can be shed from a rolling leukocyte through tethering interactions with ligands expressed on the vasculature, shedding as a result of hydrodynamic shear forces alone has not been investigated. The culture medium of Jeg-3 cells exposed to hydrodynamic shear forces was evaluated for shed L-selectin, and an insignificant amount was found. *This is a significant and novel finding because of the possible clinical treatments that could be implemented with the use of flow, in vitro, to stimulate L-selectin expression in a developing blastocyst prior to transfer.*

It has been demonstrated that the initial capture of leukocytes and subsequent rolling requires *critical thresholds* of shear stresses to occur [162-164]. The threshold shear stress range required to support rolling and attachment through L-selectin mediated

interactions has been quantified at 0.4 to 1.9 dynes/cm^2 [166, 168]. Integrin/ligand interactions facilitate secondary, higher strength bond formation. Depending on the concentration of the ligand (eg, fibronectin), bonding strengths ranging from 50 – 200 dynes/cm^2 have been measured in in-vitro systems [266, 269]. The mechanical aspects of L-Selectin and integrin mediated interactions in implantation events have not been quantified. In this project, two cell detachment measurement systems were built and validated for the purpose of evaluating detachment bond strengths associated with both primary and secondary adhesion events in the implantation process, which are thought to parallel those of leukocyte attachment. A parallel plate flow apparatus was used to quantify bond strengths between L-selectin ligand expressing Ishikawa cells, and an L-selectin functionalized substrate, which are thought to correspond to bond strengths of the initial or primary adhesion events in the implantation process. *The forces measured (0.5 dynes/cm^2) closely paralleled those seen for primary low strength binding of leukocytes to vascular endothelium. This information has provided, for the first time, some insight into the functional mechanics of the L-selectin adhesion system associated with initial reproductive events*. The parallel plate flow apparatus was also modified to include a QCM sensor, which is a novel addition that will allow the monitoring of cell detachment kinetics in real time.

A spinning disc cell detachment apparatus was built in our lab and validated with an electrochemical technique following a protocol first introduced by Garcia [248]. Bond strengths were measured between integrin expressing Ishikawa cells and a substrate functionalized with an integrin ligand (fibronectin). The spinning disc apparatus was

further validated for consistency of the range of forces generated at different angular velocities, and found to have acceptable reproducibility. Tests were also conducted on a substrates functionalized with a combination of fibronectin and L-selectin. Experimental results with the mixed L-selectin/fibronectin substrates support the hypothesis that within the 15 minute incubation time, L-selectin mediated integrin activation is probably not occurring, and the various adhesion strengths seen are most likely due to the range of fibronectin densities in the substrate.

CHAPTER 7: FUTURE WORK

This project has laid the groundwork for continuing research in two key areas which will provide further insight into the critical mediators of initial attachment mechanisms involved in blastocyst implantation. The biochemical aspects of this process have been characterized to a small extent, with the identification of the L-selectin adhesion system, and the integrin/ligand adhesion system, however, critical modulators for the expression of these proteins have not been explored. Furthermore, the mechanical aspects of the attachment process have not, to our knowledge, been characterized, by any other group. The suggestions listed below are focused on these two areas.

7.1 *Understanding the biochemistry of implantation*

The effect of other key biomarkers of implantation, such as the IL-6 family of interleukins, IL-1, LIF and CSF on the temporal expression of L-selectin and its ligands is not known. Using the same techniques and protocols as those used in this project, as well as the cell model established for the study of L-selectin/ligand adhesion system, the *effects of these important proteins on the L-selectin/ligand adhesion system could be elucidated. Additionally, the effects of endogenous and exogenous molecules, thought to be relevant factors in infertility, such as testosterone, leptin, and the inflammatory cytokine, TNF-α could be elucidated, in a manner similar to the work reported in Chapter 4 with dexamethasone.*

There are five well characterized ligands for L-selectin: GlyCAM-1, PSGL-1, CD34, MadCAM-1 and podocalyxin. The first four contain sulfated sialyl Lewis x

oligosaccharide moieties, and may be detected by the MECA 79 antibody, however, this antibody is still non-specific in that it detects only a *class* of ligands with this definitive property. MECA 79 reactive ligands as well as one specific ligand, MadCAM-1, have been found on the Ishikawa uterine epithelial cell line. MECA 79 reactive ligands have also been seen on uterine epithelial tissue by other researchers [141], and has been detected by immunohistochemistry in our lab [383]. In order to further validate the Ishikawa cell line as an appropriate cell model for the implantation process, it is necessary to continue to correlate its expression characteristics those of actual the human tissue. *Thus, verifying the presence or absence of MadCAM-1 on uterine tissue would be important. Furthermore, the known L-selectin ligands have somewhat varying functions in the body. By identifying the specific L-selectin ligands present on uterine epithelial tissue, the mechanism of the ligand function could be further defined.*

It is believed that, L-selectin binding is dependant on *sulfation* of the ligand [186]. Sulfation is a post translational modification, requiring the mediation of sulfotransferase enzymes. The essential nature of sulfotransferase enzymes was demonstrated by Uchimura whose group showed that a major class of L-selectin ligands is eliminated in mice deficient in GlyNAc6ST-1 and GlyNAc6ST-2 [199]. *Thus, a more complete understanding of the mechanism of L-selectin ligand expression in uterine epithelial tissue would be gained by studying the function of the sulfotransferase enzymes in the Ishikawa cell line.*

In this project, an IRB protocol for the collection of human uterine tissue was established and approved (Protocol #15822). To date the human biopsy tissue collected has been

preserved and evaluated immunohistologically. However, the storage protocol for the retrieved tissue could be modified such that 3D epithelial culture systems, consisting of both stromal and epithelial cells could be established. This system would allow cells to organize into structures recapitulating their *in vivo* geometry and provide a cell-based model to study the interactions of endometrial epithelium and trophoblast cells in a more biologically relevant context. Briefly, the protocol for the establishment of the primary cell culture would follows that which has been established by Satyaswaroop [384]. Primary cells are cultured in phenol red free media under estrogen-free conditions and evaluated by immunofluorescence to confirm maintenance of cell specific markers. For co-cultures, Matrigel coated gold surfaces are seeded first with human primary endometrial stromal cells which are allowed to attach. After attachment of the stromal cells, endometrial epithelial cells are seeded over the stromal cells. For analysis of biological and morphological changes, fluorescence and confocal microscopy can be used. *This 3-D endometrial epithelium culture system offers a powerful system to model early events in implantation, identify molecular pathways regulating changes in tissue architecture, and evaluate therapeutic agents in a more physiological setting.*

7.2 Quantifying the mechanical aspects of implantation

In this project, equipment has been developed and validated, and protocols have been established to quantify the attachment forces related to the L-selectin and integrin adhesion systems in an implantation cell model consisting of trophoblast-like Jeg-3 cells and uterine epithelial-like Ishikawa cells. Attachment/detachment strengths of Ishikawa

cells on L-selectin as well as integrin ligand substrates have been quantified. These studies can be greatly expanded to facilitate a functional understanding of expression levels in the L-selectin and integrin adhesion systems and their regulation by endogenous or exogenous hormones, and allow further investigation into regulation by cytokines and inflammatory mediators. Additionally, the upregulation of L-selectin on Jeg-3 cells in response to fluid flow has been shown.

For the spinning disc cell detachment apparatus, integrin/ligand interactions could be *temporally quantified with a focus on the integrin biomarkers of implantation,* $\alpha_v\beta_3$ *and* $\alpha_4\beta 1$. Further, the effects of *endogenous or exogenous hormones, cytokines and inflammatory mediators* could be quantified, for the first time.

The parallel plate flow apparatus, offers a powerful system for quantifying the critical attachment strength necessary to stabilize a blastocyst in an environment of fluid flow through L-selectin mediated adhesion interactions. With the addition of the QCM sensor, these interactions can be monitored in real time, an advantage which significantly reduces the time required for post experimental analysis. *The first task to facilitate the full use of this sensor is to quantitatively characterize the cell detachment process in terms of the sensor signal output. With the Jeg-3 trophoblast-like cells, the effects of endocrine mediators (progesterone and estrogen) and steroid hormones (eg, dexamethasone) on L-selectin shedding, capping and attachment mechanics under static and shear force conditions could be quantified using functionalized substrates of varying complexity from the sialy Lewis x tetrasaccharide molecule, to cultured Ishikawa cells, and finally to the optimized 3D endometrial epithelial cultures.* Trophoblast cells could be tested individually,

or in a spherical blastocyst-like configuration, produced in accordance with the protocol used by Tinel, et al. [304]. In figure 46, a schematic of the proposed experimental design for examination of attachment mechanics in a parallel plate flow chamber is shown.

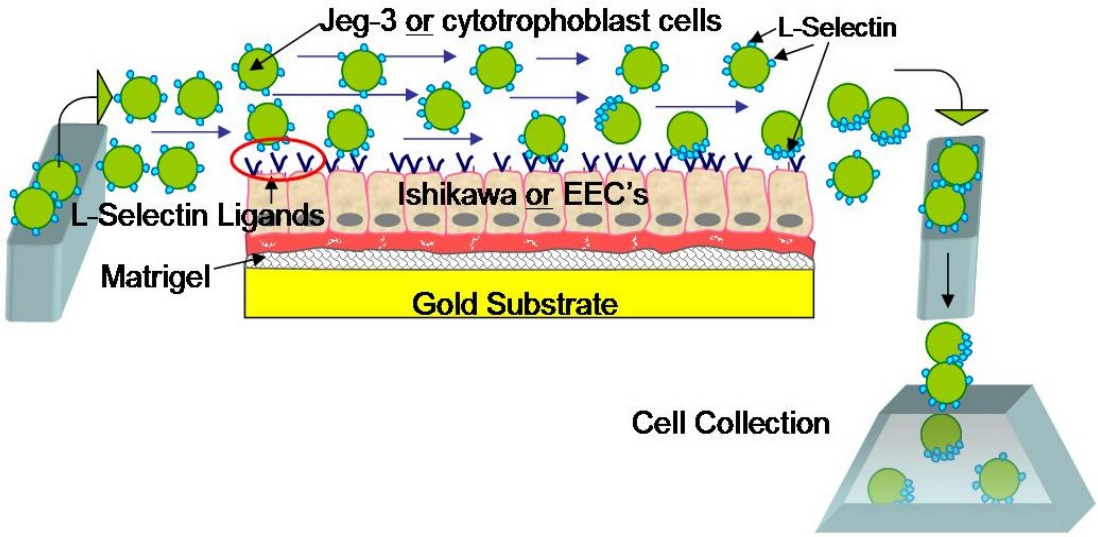


Figure 46: Schematic design of Ishikawa cell or EEC surface on a Matrigel-coated gold substrate will provide a model of the endometrial epithelium. Jeg-3 cells will either be flowed over the substrate and attachment strength will be quantified or attached to the Ishikawa or EECs and sheared off, thus quantifying detachment strength.

Conditioned or control Jeg-3 cells, collected after exposure to hydrodynamic shear (attached cells from the substrate or un-attached cells from the collection chamber) could be analyzed for L-selectin expression and capping. Cell-surface exposed L-selectin could be detected and quantified by flow cytometry. Capping and spatial and temporal redistribution of L-selectin on the cell surface of Jeg-3 cells could be imaged through confocal microscopy. Retained fluid could also be tested for L-selectin shedding using the specific ELISA established in our laboratory. Protein expression of L-selectin post

treatment could be quantified by immunoblot and by immunohistochemistry as described in Chapter 3, using the Dreg-56 L-selectin-specific antibody.

While the cell models and experimental techniques employed here are extremely useful in characterizing L-selectin and integrin mediated interactions, they are limited in the extrapolation of this data to the human implantation condition. Future research will build on this work to advance strategies for enhancing the initial embryonic attachment event in women undergoing fertility treatments.

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APPENDIX A
Protocol for Cell Passaging

A. Adherent Cell Lines

1. Equipment and Materials

   i. Ishikawa (uterine epithelial) cells

   ii. Jeg-3 (trophoblast) cells

   iii. BeWo (trophoblast) cells

   iv. JAR (trophoblast) cells

   v. 75 cm^2 cell culture flasks with vented caps

   vi. Sterile pipettes

   vii. Sterile 15 ml centrifuge tubes

   viii. Phosphate buffered saline solution (PBS)

   ix. Sanitizing disposal solution (betadine or bleach)

   x. Culture medium for Jeg-3 and Ishikawa cell lines: Eagle's Minimum Essential Media (MEM), #30-2003 (*ATCC)* with 10% Fetal Bovine Serum (FBS)

   xi. Culture medium for BeWo cell line: Kaighn's Modification of Ham's F-12 Medium (F-12K) #30-2004 (*ATCC)* with 10% Fetal Bovine Serum (FBS)

   xii. Culture medium for JAR cell line: RPMI-1640 Medium, #30-2001 (*ATCC)* with 10% Fetal Bovine Serum (FBS)

   xiii. Centrifuge

xiv. Incubator maintained at 37°C, with 5% CO_2

2. Cell cultures of *JAR, Jeg-3 and Ishikawa* cells are ready to be passaged (split) when they are 70-100% confluent.
3. For *BeWo* cells, passaging should be done when cultures reach 60-80% confluency.
4. With a sterile pipette, remove culture medium and discard in sanitizing disposal solution
5. For *BeWo, JAR and Jeg-3* cell lines, briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution to remove all traces of serum (which contains trypsin inhibitor).
6. For *Ishikawa* cells, briefly rinse the cell layer with PBS
7. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to the flask and incubate at 37°C for 5-15 minutes. (*Periodically observe cells under an inverted microscope. When cell layer is separated into small aggregates, proceed to step 8. DO NOT agitate cells during this incubation period)*
8. Add 9.0 ml of FBS supplemented culture medium and separate cells by gently pipetting, and lightly tapping the flask to provide aggitation.
9. Remove all culture medium with inactivated trypsin and cell suspension by pipette, and transfer to a sterile 15 ml centrifuge tube.
10. Pellet the cells by centrifuging at 1000 RPM for 5 minutes.
11. Pour off supernatant, and resuspend the cells in 10 mls of fresh FBS supplemented culture medium.

12. Add appropriate aliquots of the cell suspension to new culture vessels:
    i. Split ratio for Ishikawa and Jeg-3 cell lines ~ 1:10
    ii. Split ratio for JAR cell line ~ 1:20
    iii. Split ratio for BeWo cell line ~ 1:5
13. Add enough fresh FBS supplemented culture medium to the flask to achieve a total volume of 15 mls.
14. Incubate cultures at 37^{o}C to recommended confluency levels before splitting.

B. Non-adherent Cell Line

1. Equipment and Materials
    i. U-937 cell line
    ii. Sterile pipettes
    iv. Sanitizing disposal solution (Betadine or bleach)
    v. Culture medium for U-937 cell line: RPMI-1640 Medium, #30-2001 (*ATCC)* supplemented with 10% Fetal Bovine Serum (FBS)
    vi. Incubator maintained at 37^{o}C, with 5% CO_2
2. Cells should be split when they reach an ~ concentration of 1 – 2 x 10^6 cells/ml
3. Remove 13. 5 mls of the cell suspension and discard in sanitizing disposal solution.
4. Add 13.5 mls of fresh culture medium supplemented with 10% Fetal Bovine Serum (FBS).

5. Incubate at 37^{o}C in 5% CO_2 until cells reach a concentration of $1 – 2 \times 10^6$ cells/ml.

APPENDIX B
Hormonal Conditioning of Cell Cultures

A. Equipment and Materials

1. Estradiol (10^{-5} M) in ethanol (stock)
2. Progesterone (10^{-3} M) in ethanol (stock)
3. Dexamethasone (10^{-4} M) in ethanol (stock)
4. Sterile phosphate buffered saline solution (PBS)
5. Sterile pipettes
6. Sterile phosphate buffered saline solution (PBS)
7. Sterile centrifuge tubes
8. Sanitizing disposal solution (Betadine or bleach)
9. Phenol red free culture medium, supplemented with 10% charcoal stripped fetal bovine serum (FBS):
    i. *JAR and U-937* cell lines: RPMI-1640, *#MT-17-105CV (Fisher Scientific*)
    ii. *Ishikawa, Jeg-3 and BeWo* cell lines: D-MEM F-12, *#21041(Gibco)*

B. Conditioning Protocol

1. Passage cells according to standard protocol, and allow to incubate for 24 hrs. to assure that cells are in log growth phase.
2. For adherent cell lines, remove culture medium by pipette, and discard. Follow steps 3-6.

3. Wash adherent cells with 5-10 mls of PBS 1X, remove PBS by pipette, and discard.
4. Replace original culture medium with similar amount of FBS supplemented phenol red free culture medium.
5. Add desired stock hormone solution to test flask in 1:1000 concentration
6. Incubate conditioned cultures at 37^{o}C (5% CO_2) for 24 hours before collection and cell lysate preparation.
7. For non-adherent cell line, follow steps 8-14.
8. Remove cell suspension and place in appropriately sized centrifuge tube.
9. Pelletize the cells by centrifuging for 5 minutes at 1000 RPM.
10. Pour off supernatant
11. Resuspend cells in 10-20 mls of PBS, aspirating gently to assure complete washing.
12. Pelletize the cells by centrifuging for 5 minutes at 1000 RPM.
13. Pour off supernatant.
14. Resuspend cells in an amount of FBS supplemented phenol red free culture medium similar to the original amount of culture medium.
15. Add desired (stock) hormone solution to test flask in 1:1000 concentration
16. Incubate conditioned cultures at 37^{o}C (5% CO_2) for 24 hours before collection and cell lysate preparation.

APPENDIX C
Protocol for Cell Lysate Preparation

This protocol was developed for the preparation of cell lysates from Ishikawa, JAR, Jeg-3 and BeWo cells. The lysates were intended to be analyzed for L-selectin and L-selectin ligands.

A. Lysis Buffer

1. Equipment and Materials

    i. Molecular grade water

    ii. Tris-acetate (1M) solution in water (stock)

    iii. Calcium chloride (CaCl) (0.5M) solution in water (stock)

    iv. 10% NP-40 solution in water (stock)

    v. Sodium orthovanadate (100 mM) in water (stock)

    vi. Pepstatin (1g/ml) in water (stock)

    vii. Sodium fluoride (NaF) (1M) in water (stock)

    viii. Chymostatin (10 mM) in Dimethyl sulfoxide (stock)

    ix. Benzamidine HCl (10mg/ml) in water (stock)

    x. "Complete Mini" protease inhibitor cocktail tablets (*Roche*)

    xi. Ice

2. Add 9.6 mls of molecular grade water to a 15 ml conical centrifuge tube (or similar container)
3. Add 100 uL of stock Tris-acetate to the water
4. Add 100 uL of stock CaCl to the water

5. Add 100 uL of stock NP-40 to the water
6. Mix well and place the solution on ice to cool for ~15 minutes
7. Add 100 uL of stock sodium orthovanadate to the solution
8. Add 10 uL of stock NaF to the solution
9. Add 10 uL of stock pepstatin to the solution
10. Add 1 “complete mini” tablet to the solution
11. Mix the solution to dissolve the tablet: keep cold
12. Add 50 uL of stock chymostatin to the solution
13. Add 10 uL of stock benzamidine HCl to the solution
14. MIX WELL and keep cold: store at 4^{o}C for no longer than one week

B. Collection of Cells and Lysate Preparation

1. Equipment and Materials
    i. Microcentrifuge tubes
    ii. Lysis buffer
    iii. Ice
    iv. Syringes with 22 gauge needle or higher
    v. Cell scrapers
2. NOTE: All steps should be performed at 4^{o}C, or as close as possible to this temperature
3. Remove culture/conditioning medium from culture flask
4. Wash the adherent cells 1X with sterile PBS (2 – 10) mls depending on the flask size)

5. Remove PBS by suction; allow all PBS to drain to the bottom of the flask, and maintain suction until all is removed before adding the lysis buffer.
6. Add sufficient lysis buffer to the flask to assure a complete layer of coverage (200-500 uL, depending on the flask size
7. After 5 minutes, begin scraping the cells from the flask surface.
8. Transfer cells in lysis buffer to a microcentrifuge tube, keep cold.
9. Incubate cells in lysis buffer for 30 minutes; vortex the solution about every 10 minutes.
10. Lyse cells by drawing the solution into a syringe then pushing the solution out forcibly.
11. Repeat step 9 at least 10X.
12. Centrifuge cell/lysate solution for 5 minutes at 14, 000 ppm, in a refrigerated centrifuge at 0^{o}C, if possible.
13. Transfer lysate to a new container, discard pelleted cellular debris.
14. Measure total protein content of the lysate via BCA (bicinchoninic acid) protein assay, following manufacturers instructions.
15. Store lysates at -80^{o}C until prepared for gel electrophoresis.

APPENDIX D
Protocol for Western Blot (reducing conditions) for L-selectin and L-selectin ligands

A. Gel Electrophoresis

1. Equipment and Materials:

    i. Gel Electrophoresis Apparatus, #170-3836 Mini-PROTEAN Trans-Blot cell (*Bio-Rad*)

    ii. Running Buffer: 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS, # 161-0772, 10X (*Bio-Rad*)

    iii. SDS-PAGE Gels, #161-1105 (*Bio-Rad*)

    iv. Protein Standard #161-0374 (*Bio-Rad*)

    v. Cell Lysates

    vi. Laemmli buffer #161-0737 (*Bio-Rad*)

    vii. Power supply

2. Prepare cell lysates, and determine total protein content
3. Mix cell lysates in a 1:1 ratio with laemmli buffer(with β-mercaptoethanol added per instructions)
4. Heat @ 90-100 ^{o}C for 5 minutes
5. Allow lysate/laemmli buffer mixture to cool to room temperature.
6. Prepare the running buffer according to instructions.

7. Assemble the SDS-PAGE gels into the Gel Electrophoresis Apparatus per instructions. Add enough running buffer to the internal compartment to just cover the gel completely. Add a similar amount to the external compartment.
8. Determine the amount of protein lysate mixture (uL) that must be added to each well such that all wells contain the same amount of *total* protein sample.
9. Load the wells with lysate sample, controls and standards, and add additional running buffer to both compartments to fill the container.
10. Hook the electrodes to the power supply and set the voltage to 100V for 1 to 1.5 hrs, depending on the separation efficiency. (Note: A good connection has been made when small bubbles are observed rising from the bottom of the chamber).
11. After the separation is complete remove the gels from the chamber and assemble them into the cassette for the (Western Blot) transfer process (Step B).

B. Western Blot

1. Equipment and Materials:
    i. Mini Trans Blot Module, #170-3935 (*Bio Rad*)
    ii. Hybond ECL nitrocellulose sheets, # NC9228451 (*ThermoFisher Scientific*)
    iii Thick blot filter paper, #170-3932 (*Bio Rad*) (4 pieces)
    iv Transfer buffer: Tris, glycine, methanol, water, #161-0771 (*Bio Rad*, 10X)

v. Power supply

vi. Phosphate buffer solution (PBS)

vii. Tween 20

viii. Primary antibody: DREG 56, # 304811 (*Biolegend*) MECA-79, #553863 (*BD Pharmingen*) or anti-MadCAM-1, # MCA2096Z and # MCA23 (*AbD Serotec*)

ix. Secondary antibody: HRP conjugated

x. Non-fat powdered milk

xi. Laboratory rocker or orbital platform shaker

xii. ECL+ Western Blot Detection system #32106 (*Pierce Chemicals*)

xiii. Chemiluminescence film, #RPN3114K (*GE Amersham)*

2. Prepare transfer buffer according to instructions, and place some in a soaking dish
3. Cut two nitrocellulose membranes approximately the same size as the gels.
4. Soak the nitrocellulose membranes, filter paper, and gauze in transfer buffer at least 10 minutes before assembling into the module.
5. Remove the gel from its glass housing and place on top of the wetted nitrocellulose membrane, in the transfer buffer.
6. Stack the gel, nitrocellulose sheet, filter papers and gauze pads as shown in Figure 1, taking care not to trap bubbles between the layers.

7. Place the stack (from step 6) in the holder as shown in Figure 1. Make sure that the SDS-PAGE gel is closest to the black side of the holder, and the nitrocellulose sheet is closest to the white side of the holder.

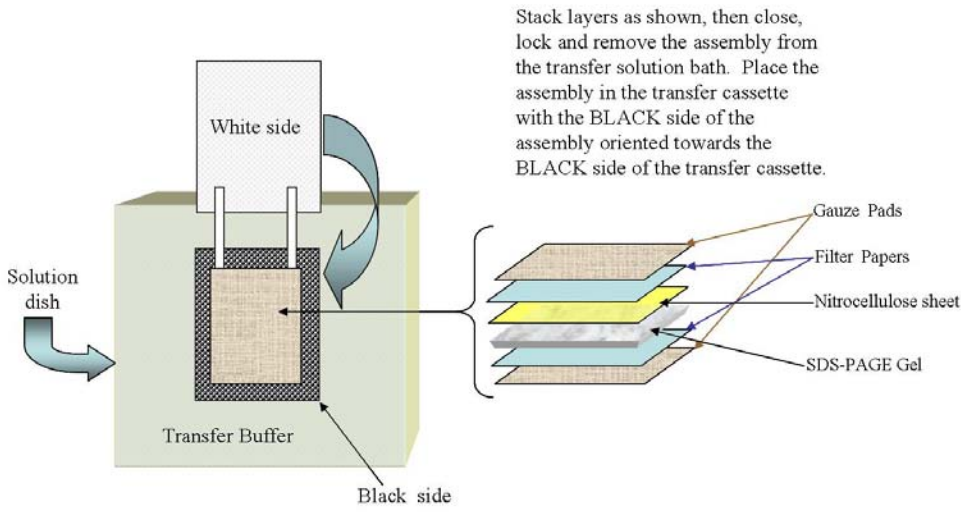

Figure 1: Setup of gel and nitrocellulose sheet for electrophoretic transfer

8. Place the holder in the western blot transfer cassette, oriented such that the black side of the \holder is towards the black side of the cassette.
9. Place the cassette into the tank, along with a stirring bar and the ice pack.
10. Fill the tank with transfer buffer, place the top on the tank and hook the electrodes into the power supply.
11. Set the power to 100 V for 1 hour, agitate the solution with the stirring bar.

12. At the end of one hour, disassemble the transfer cassette, and remove the nitrocellulose sheets (These now have the separated proteins on the surface).
13. Block non-specific binding sites on the nitrocellulose sheets with 7% (w/v) non-fat powdered milk in PBS+0.1% Tween (PBST) for 1 hour at room temperature, or overnight at 4^{o}C. Be sure to agitate.
14. Incubate the nitrocellulose sheets in the primary antibody diluted as recommended in 5% (w/v) non-fat powdered milk in PBST for 1 hour at room temperature, or overnight at 4^{o}C. Be sure to agitate.
15. Rinse the nitrocellulose sheets briefly 2x with PBST, then wash for 15 minutes in PBST with agitation.
16. Wash 2x in PBST for 5 minutes each.
17. Incubate the nitrocellulose sheets in the secondary antibody diluted as recommended in 5% (w/v) non-fat powdered milk in PBST for 1 hour at room temperature, or overnight at 4^{o}C. Be sure to agitate.
18. Rinse the nitrocellulose sheets briefly 2x with PBST, then wash for 15 minutes in PBST with agitation.
19. Wash 1x in PBST for 5 minutes, followed by 1x in PBS for 5 minutes. Visualize protein blots with ECL+ Western blot detection system, per instructions.

APPENDIX E
Immunohistochemistry of Ishikawa Cells

A Materials

1. 4% Paraformaldehyde solution
2. 4-well Permanox chamber slides #12-565-21 (*Fisher Scientific*)
3. Phosphate buffered saline solution (PBS)
4. Phosphate buffered saline solution + 0.05% Tween 20 (PBS-T)
5. Blocking Solution: 10 % Normal Goat Serum (NGS) in PBS
6. Diluent: 5 % Normal Goat Serum (NGS) in PBS
7. Streptavidin/Biotin blocking Kit, #SP 2002, (*Vector Labs*).
8. β-Actin rabbit polyclonal anti-actin antibody (*Cell Signaling Technology, Inc*.): 5uL /250 uL of diluent
9. Biotinylated anti-rabbit IgG # BA-1000 (*Vector Labs*): 5uL /250 uL of diluent
10. MECA-79 rat anti-mouse monoclonal antibody (*BD Pharmingen)*: 5uL /250 uL of diluent
11. Rat IgM isotype control #14-4341 (*eBioscience*) : 5uL /250 uL of diluent
12. Biotinylated anti-rat IgM Antibody, #16-16-03 (*KPL*) (~700 uL, 0.5 mg/ml)
13. Alkaline phosphatase streptavidin, #SA-5100 (*Vector Labs)*
14. BCIP/ NPT/alkaline phosphatase substrate kit #SK-5400 (*Vector Labs*)

B. Protocol

1. Seed Ishikawa cells in 4-well Permanox chamber slides and incubate in normal culture medium for 24 hrs. at 37^{o}C, in 5% CO_2
2. Remove culture medium and wash 1X with PBS.
3. Fix cells with 4% paraformaldehyde solution for 30 minutes at room temperature.
4. Remove paraformaldehyde and wash 1X with PBS.
5. Block for 1 hour at room temperature in 10% NGS in PBS.
6. Wash 1X with PBS-T
7. Block for 15 minutes with streptavidin D solution
8. Wash 1X with PBS-T
9. Block for 15 minutes with biotin solution
10. Wash 1X with PBS-T
11. Add primary antibody (MECA-79, anti-actin, or rat IgM isotype), and incubate at 4^{o}C overnight.
12. Wash 3X with PBS-T
13. Incubate in corresponding biotinylated secondary antibody at room temperature for 1.5 hrs.
14. Wash 1X with PBS-T
15. Incubate at room temperature for 1.5 hrs. in alkaline phosphatase streptavidin diluted in 10% NGS in PBS-T.
16. Wash 2X with PBS-T

17. Wash 1X with PBS
18. Prepare BCIP solution per directions. Add to each well, followed by 1 drop of levamisole.
19. Incubate at room temperature until a color change is observed.
20. Wash 2X with DI water and allow slides to dry.
21. Add 1-2 drops of aqueous mounting solution and a coverslip to seal slide
22. Analyze for staining intensity.

APPENDIX F
Protocol for Enzyme-Linked ImmunoSorbent Assay (ELISA) of Shed L-Selectin

This protocol was developed for ELISA analysis of L-Selectin shed into culture medium of conditioned Jeg-3 and Ishikawa cells.

A. Concentration of culture medium

1. Equipment and materials
    i. Protein media concentrators: Microcon YM-30 and Centriplus YM-30 (*Millipore Corp*)
    ii. Syringes with 0.2 μm low-protein binding syringe filter
2. NOTE: For all steps, the temperature of the culture medium should be kept at -4^{o}C as much as is practically possible.
3. Collect culture medium from cell culture flask, cool on ice.
4. Centrifuge at 1000 g for 10 minutes
5. Filter cooled culture medium through syringe filters
6. Place up to 15 ml of filtered culture medium in Centriplus protein concentrator (measure volume precisely, and note as initial volume).
7. Centrifuge according to manufacturers recommendations to a volume of ~ 1000 – 500 uL.
8. Transfer concentrated culture medium (500 uL max) to YM-30 Microcon protein concentrator, and centrifuge according to manufacturers recommendations, to a final volume of ~200-300 uL.

9. Carefully measure final volume, and calculate concentration factor.

10. Store concentrated culture medium at -80^{o}C until use.

B. ELISA

1. Equipment and materials

   i. Coating buffer - 100 mN Bicarbonate/carbonate buffer: 3.03 g Na_2CO_3, 6.0 g $NaHCO_3$ in 1L of distilled water, adjusted to pH 9.6.

   ii. Blocking buffer - 3% (w/v) BSA in PBS

   iii. Wash buffer – PBS + 0.1% (v/v) Tween-20 (PBS-T)

   iv. Stopping buffer – 3 M HCl

   v. ELISA 96-well plates

   vi. Polyclonal L-selectin (capture) antibody #N-18, (*Santa Cruz Biotechnology*)

   vii. DREG 56 L-selectin (primary) antibody, #304811 (*BioLegend*)

   viii. Biotinylated goat anti-mouse (secondary) antibody #BA-9200 (*Vector Labs*)

   ix. HRP conjugated streptavidin #21126 (*Pierce*)

   x. TMB (tetramethylbenzidine) substrate reagent set, #1854050 (*Pierce Chemicals*)

2. Coat 96-well ELISA plates with 100 µL/well of 3 µg/ml polyclonal L-selectin (capture) antibody (N-18) in coating buffer at 4^{o}C overnight

3. Wash the plate 2X in PBS

4. NOTE: firmly blot the plate against clean paper towels at the end of each washing step.
5. Add 200 µL/well of blocking buffer and incubate for 2 hours at room temperature with shaking.
6. Wash the plate 3X in PBS-T
7. Prepare L-selectin standards in culture medium
8. Add 100 µL/well of concentrated culture medium, standards and blank to the plate
9. Incubate for 2 hours at room temperature with shaking.
10. Wash the plate 5X in PBS-T
11. Add 100 µL/well of DREG 56 antibody (1:500 in blocking buffer) to the plate.
12. Incubate for 1 hour with shaking at room temperature
13. Wash the plate 5X in PBS-T
14. Add 100 µL/well of biotinylated goat anti-mouse secondary antibody (4 µg/ml in blocking buffer) to the plate
15. Incubate for 1 hour with shaking at room temperature
16. Wash the plate 5X in PBS-T
17. Add 100 µL/well of streptavidin-HRP (0.5 µg/ml in blocking buffer) to the plate
18. Incubate for ½ hour with shaking at room temperature
19. Wash the plate 5X in PBS-T

20. Mix TMB reagents (1:1) and add 100 µL/well to the plate
21. Cover the plate with a material impervious to light (eg, aluminum foil).
22. Incubate for ½ hour with shaking at room temperature
23. Add 100 µL/well of stopping buffer to the plate to stop the reaction (DO NOT REMOVE TMB REAGENTS)
24. Measure absorbance at 450nm with a microplate reader

APPENDIX G
Protocol for L-selectin Attachment to Gold via Self Assembled Monolayer (SAM) Linker

A. Cleaning of Gold Surface

1. Materials

   i. 30 % Hydrogen peroxide (H_2O_2) solution

   ii. 25% Ammonia (NH_3) solution

2. Prepare a solution of hydrogen peroxide:ammonia:water in a ratio of 1:1:5

3. Heat the solution to 80^{o}C

4. Immerse the gold substrates in the solution for 5-10 minutes.

B. Deposition of Mixed SAM

1. Materials

   i. Absolute ethanol

   ii. 11-Mercaptoundecanoic Acid (MUA): $HS(CH_2)_{10}COOH$ F.W. 218.36

   iii. 1-Decanethiol (NDt): $HS(CH_2)_9CH_3$ F.W. 174.35

2. Prepare a binary mixture of 2.5 mM MUA and 7.5 mM NDt in absolute ethanol

3. Immerse the clean gold surface in the mixture for three (3) hours at RT

4. Thoroughly rinse in absolute ethanol, and dry in a flow of clean air

C. Covalent immobilization of L-selectin

1. Materials:

    i. Ultrapure water

    ii. N-3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC): $C_8H_{17}N_3$ F.W. 155.24

    iii. N-Hydroxysuccinimide (NHS): $C_4H_5NO_3$ F.W. 115.09

    iv. L-selectin Fc Chimera (R&D Systems) 25 ug/ml in 10 mM PBS @ pH 7.4

    v. ethanolamine (1M) @ pH 9.0

    vi. 1% (w/v) BSA in 10 mM PBS @ pH 7.4

2. Prepare a solution of 20mM NHS and 10 mM EDC in ultrapure water.
3. Immerse the SAM coated gold substrate in the above solution for 2 hours at RT to "activate" the SAM
4. Prepare a solution of L-selectin Fc Chimera at a concentration of 25 ug/ml in 10 mM PBS @ pH 7.4
5. Immerse the "activated" SAM coated substrate in the L-selectin solution for 2 hours at R.T
6. Block any remaining active sites by immersion in the ethanolamine solution for 20 minutes at R.T
7. Wash the coated gold substrate thoroughly with ultrapure water (~ 4-5 rinses)
8. Immerse the gold substrate in BSA solution for 2 hours.

Vita

Dianne M. (Phelan) Rothstein was born in Philadelphia, PA in October of 1953. She attended Indiana University of Pennsylvania and graduated in June, 1975 with a Bachelor of Science degree in Biology, and a minor in Chemistry. Dianne was employed at Leeds & Northrup Co (L&N), North Wales PA from 1975 to 1991, starting as a Research Department Chemistry Technician, and advancing to Senior Scientist and Electrochemistry Group Leader. During this period, Dianne was responsible for the development of liquid analysis sensors for fermentation process control, water treatment, part per billion (ppb), and medical applications. Dianne served on L& N's President's Council as well as L&N's Safety Committee. Dianne received her Masters of Science Degree in Chemistry from Drexel University, Philadelphia, PA in June, 1984. Dianne served as President/Co-owner of Omni Builders as well as a Real Estate Sales Associate from 1991 to 1994. She joined Capital Controls Co. in Colmar, PA as a Senior Chemist in August, 1994 where she was responsible for project management and technical support for all chemistry related issues and on-line liquid analysis equipment. She also served on American Water Works Association (AWWA) Standard Methods review committee, and as Co-Chair on Capital Controls Safety Committee during this time. Dianne attended Drexel University from 1999 to 2007 pursuing a PhD in Materials Science. Since 2004, Dianne has also been employed by Prime Synthesis, Inc, Aston, PA, as Vice President of Research. Dianne has 9 publications and presentations, as well as 1 patent entitled "Method of Measuring Chlorine Content in Aqueous Solutions".